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The Influence Of Site Connectivity On Zooplankton Assemblage Dynamics Within The Lower Mississippi River Floodplain

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THE INFLUENCE OF SITE CONNECTIVITY ON ZOOPLANKTON ASSEMBLAGE DYNAMICS WITHIN THE LOWER MISSISSIPPI RIVER FLOODPLAIN

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ABSTRACT

Zooplankton are important components of the food web of such lotic systems. Although capable of motility, they are weak swimmers and their distribution and community composition across a floodplain is strongly affected by patterns of water flow and hydrologic connectivity. However, there has been little research on the zooplankton dynamics of large rivers, such as the Lower Mississippi River (LMR). From morphological identification of zooplankton across the connectivity gradient, I assessed the degree to which hydrologic connectivity acts as an explanatory factor influencing spatial and temporal variation in zooplankton assemblage structure. Second, I compared the use of morphological identification to identification by molecular metabarcoding using Illumina sequencing of the cytochrome oxidase subunit I (COI) gene. Finally, I examined the spatial distribution of haplotypes of three zooplankton species (*Anuraeopsis sp. 2017a*, *Diaphanosoma sp. 1*, and *Leptodiaptomus siciloides*) and tested for evidence of cryptic species in two additional zooplankton species (*Keratella cochlearis*, and *Brachionus calyciflorus*). Rotifer assemblages across the sampling area were most correlated with combinations of dissolved nitrogen, turbidity, temperature, and chlorophyll a. Crustacean assemblages were most correlated with combinations of dissolved nitrogen, turbidity, temperature, chlorophyll a, and dissolved oxygen. Temperature was correlated with all beta diversity measures emphasizing a seasonal succession of zooplankton within the LMR. In general, barcoding assemblages is useful for defining patterns in zooplankton across connectivity

as a large amount of data can be collected in a relatively short period of time compared to morphological identification. However, at this time, species abundance cannot be determined using Illumina sequencing data. Spatial distribution of haplotypes varied across the three species examined. Finally, one (*Brachionus calyciflorus*) of the two species tested for cryptic species showed strong agreement between the three methods used, while *Keratella cochlearis* had no agreement between methods. These results indicate that there may be either physiological limitations to cryptic species members morphologically identified as *Keratella cochlearis* from across the LMR catchment, causing those members to be excluded in the sample area, or that this region of the COI is relatively conserved across cryptic species of *Keratella cochlearis* within the sample area.

DEDICATION

To everyone who supported me along the way.

LIST OF ABBREVIATIONS AND SYMBOLS

ABGD	Automatic barcode gap discovery
BOLD	Barcode of Life Database
BPA	Borrow pit A
BPB	Borrow pit B
bPTP	Bayesian implementation of the Poisson tree process model
Chla	Chlorophyll *a*
COI	Cytochrome oxidase subunit I
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
Eu	Eupotamal
GH	Gloryhole Lake
GMYC	Generalized mixed Yule coalescent approach
GY	Graveyard Lake
I63	Island 63 secondary channel
I64	Island 64 secondary channel
JS	Jim Samples Lake
LDN	Lower Desoto Lake – North
LDS	Lower Desoto Lake – South

LMR	Lower Mississippi River
LMW	Lower Mellwood Lake
McW	McWilliams Lake
MDN	Middle Desoto Lake – North
MDS	Middle Desoto Lake – South
MMW	Middle Mellwood Lake
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multidimensional scaling
ORC	Old River Chute
Paleo	Paleopotamal
Para	Parapotamal
PCR	Polymerase chain reaction
Plesio	Plesiopotamal
PN	Particulate nitrogen
POC	Particulate organic carbon
SFD	Sunflower dike field
Temp	Temperature
TDN	Total dissolved nitrogen
TDOC	Total dissolved organic carbon
Turb	Turbidity

UD	Upper Desoto Lake
UMW	Upper Mellwood Lake
ZOTU	Zero-radius operational taxonomic unit

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CHAPTER I: INTRODUCTION

1.1 Hydrologic Connectivity of Large River Systems

Ward (1989) described the interrelation of a lotic system in four dimensions. The dimensions are represented as longitudinal (upstream to downstream movement), lateral (main channel/floodplain exchange), vertical (interactions of river/groundwater), and temporal (change over time). This framework was expanded upon by Tockner et al. (2000) who defined hydrologic connectivity in terms of fluxes of water, associated organisms and other materials across these four dimensions. The concept of hydrologic connectivity is now recognized as a driving force of ecological processes in river systems (Junk et al. 1989; Hein et al. 2001; Pringle 2003).

Unless it has been fully channelized, a large river system will consist of the main channel and various forms of floodplain backwaters, including lakes, wetlands, and secondary channels. With expansion of the river during high river stages, connections between the main channel and these backwaters are strong, facilitating fluxes across the system of organisms, nutrients, and organic materials (Tockner et al. 1999a). The main stem of a river provides an abundance of available nutrients to floodplain backwaters, which can increase primary production (Cloern 2007), but can also profoundly change

the physical and chemical nature of backwaters (Bayley 1995; Amoros and Bornette 2002). As the river contracts with a decline in river stage, lateral fluxes of materials (e.g. sediments, nutrients), as well as the corridor for large animal dispersal, diminishes until a critical point where surface hydrologic connection between river channel and floodplain backwaters disappears. This critical point can be different for each floodplain site and may be reached and breached several times throughout each year. Below the critical point, stage height is conferred to backwaters through groundwater connection only, if at all. Thus, changes in the degree of connectivity can have strong direct and indirect effects on the biota that occupy both backwater and main channel habitats.

1.2 The Role of Hydrologic Connection in Large River Ecological Processes

Ward et al. (1999) made use of a modified dynamic equilibrium model (Huston 1979) to describe biodiversity changes within rivers. According to this model, highest biodiversity is predicted in habitats with "matching" levels of disturbance and resources. Habitats with low productivity and high disturbance are characterized by a few species with high growth rates/short generation times leading to low diversity. Habitats with high productivity and low disturbance are subject to high rates of competitive exclusion leading to low diversity. Gallardo et al. (2008) found support for this model showing a unimodal distribution of macroinvertebrate diversity across a connectivity gradient in the Ebro River, Spain. However, amphibian diversity has been reported to increase with decreasing connectivity (Tockner et al. 1999b). Diversity is not the only biotic characteristic affected by connectivity. Roach et al. (2009) found that trophic position of three zooplanktivorous fish species increased in backwater habitats compared to more connected

habitats. However, organisms that inhabit the largest rivers remain understudied and it is unclear how the seasonal changes in connectivity of riverine landscapes affect biota at this scale.

1.3 Effects of Connectivity on Zooplankton of Large Rivers

The term zooplankton comes from the Greek words zoon (meaning animal) and planktos (meaning drifter or wanderer). Using the simplest definition, zooplankton can be applied to many organisms that live within a water column and are unable to resist flow in a system. This characterization can include, but is not limited to, certain insects and their larvae, juvenile fish, copepods, bivalve larvae, cladocerans, and rotifers. This research focuses on the rotifer, cladoceran, and copepod components of the zooplankton assemblage which in most aquatic ecosystems tend to be the dominant taxa (Prosser et al. 2013). These taxa are all influenced by connectivity to the main channel, albeit somewhat differently.

Rotifers are smaller and reproduce more quickly than crustacean zooplankton. Thus, upon disconnection of a lake site from the main channel, both rotifers and crustacean zooplankton will increase in number but the response is slower for crustaceans (Baranyi et al. 2002; Keckeis et al. 2003). Górski et al. (2013) suggested that inundated terrestrial habitat heterogeneity following seasonal flood pulses plays an important role in structuring zooplankton assemblages in the Waikato River, New Zealand. The ecosystem focus of this dissertation, the Lower Mississippi River (LMR) is larger than the rivers of these previous studies and, in general, the biology of large rivers is understudied. Zooplankton in large rivers are themselves understudied despite their involvement in essential ecosystem processes.

Although copepods, cladocera, and rotifers each have members that can be grouped as zooplankton, they display diverse life history, feeding, and reproductive strategies which can

affect where member species are found across the landscape. Copepods exhibit sexual reproduction with males and females being dimorphic. Copepods also have multiple developmental stages, after hatching from fertilized eggs they go through six naupliar larval stages before moving through six copepodid stages, the final being a reproductive adult. Copepods are omnivorous, feeding on a variety of phytoplankton, detritus, and other zooplankton (Reid and Williamson 2010). When food becomes scarce, or when the population becomes overcrowded, diapause may be triggered in either egg or copepodid stages. Diapause ends when conditions improve allowing the population to rebound.

Cladocerans share similar life histories and feeding strategies to rotifers, although as crustaceans they are more closely related to copepods. Both cladocerans and rotifers have many species that exhibit cyclic parthenogenesis. When conditions are favorable, female cladocerans and rotifers reproduce asexually by ameiotic parthenogenesis, effectively cloning new members of the population (Dodson et al. 2010; Wallace and Snell 2010). As conditions become unfavorable, males are produced, sexual reproduction occurs and resting eggs remain unhatched until conditions improve. Although parthenogenesis is widespread in both taxa, there are members of each that reproduce strictly sexually or strictly asexually (e.g. Bdelloid rotifers). Cladocerans and rotifer assemblages are also dominated by phytoplankivorous grazers causing significant top down effects on phytoplankton (Carpenter et al. 1987).

Although different taxa have varied methods for reproduction and feeding, all zooplankton are controlled either directly or indirectly by similar biotic (Semenchenko et al. 2007) and abiotic factors. Apart from physical and chemical effects of hydrologic connectivity (which will be addressed in Chapter II), there are several biotic and behavioral influences on

zooplankton assemblage structure. Fish have long been known to structure zooplankton assemblages in clear waters (Brooks and Dodson 1965) and it has been suggested that fish predation on zooplankton is reduced in turbid waters by visual impairment of spot feeding planktivorous fish (Jack and Thorp, 2002). However, Schulze (2011) found that fish predation may be significant in turbid lakes and reservoirs since small bodied zooplankton were more common in areas with fish and large bodied zooplankton were more common in areas without fish. In rivers, both scenarios may be true since turbidity levels (Thorp and Mantovani 2005; Ochs et al. 2013) have the potential to far exceed the 15-NTU (Nephelometric Turbidity Units) that Schulze (2011) used and because of differences in behavior of zooplankton and fish between lake and riverine environments. Planktivorous filter feeding fish, including native paddlefish (*Polyodon spathula*) and invasive silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys nobilis*) of the LMR may also have a marked effect on assemblage structure (Shrank et al. 2003; Varble et al. 2007). However, fish avoidance is not the only behavioral strategy that may influence the horizontal distribution of zooplankton and zooplankton assemblage structure.

Shoreline and outflow avoidance behaviors by zooplankton may determine where they occur horizontally within a lake environment. Rindelburg (1987) suggested that crustacean zooplankton actively avoid shorelines based on light changes and Brook and Woodward (1956) found that crustacean zooplankton actively avoid the outflow of lakes. There has been a question of whether crustacean zooplankton avoid the shoreline or predacious fish that occur in littoral regions of lakes. Wicklum (1999) found that, despite a fishless environment, *Daphnia* abundance was lower in near shore areas compared to middle lake sites. Larger differences between sites

were found in lakes that contained fish compared to fishless lakes, which suggests a combination of predator and shore avoidance by *Daphnia*. Wicklum (1999) also gave support to active outflow avoidance by *Daphnia*, stating that abundances in outflow streams were lower than those found in the middle of the lake. Further support for outflow avoidance by *Daphnia* is given by the fact that rotifers and nauplii, but not adult *Daphnia*, occurred in similar abundances between outflow streams and mid-lake sites (Wicklum 1999).

1.4 The Lower Mississippi River System – A Model for Connectivity Effects

The LMR is an excellent model for studying connectivity effects across a large river system. Unlike its major tributaries (e.g. the Ohio, Upper Mississippi, and Missouri Rivers), the LMR lacks low-head and hydro-electric dams. This absence allows for a more natural rise and fall of river stage without a low stage maintained depth with low head dams or the intermittent flow regimes created by large dam structures. Although free of dam structures, the LMR has an extensive levee system that has reduced the natural floodplain to about 10% of its historic area (Baker et al. 1991). However, while there has been a drastic reduction in floodplain connectivity, a dynamic range of backwater habitats within the levee system of the LMR remains (Ochs et al. 2013). The LMR is also characterized by a large network of dikes and weirs to maintain navigation channel depth by diverting flow away from side channels and main channel margins. Although achieving the intended goal, these areas may be over-engineered to the detriment of biota that rely on reduced but continuous flow (J. Kilgore and A. Harrison-Lewis pers. comm.)

To reduce navigation channel length, the Army Corps engineered cutoffs of some large river meanders of the LMR (Baker et al. 1991; Alexander et al. 2012). These cutoffs have created large oxbow lakes that fill and drain in relation to river stage height. These large lakes

and smaller backwater lakes have a flow through from upstream to downstream at high river stages. As the river drops, the upstream connection is lost and only the downstream connection remains. Any rise or fall in river stage is then conferred to backwater lakes through a single opening, if at all (Pongruktham and Ochs 2015). Also of interest is the widespread use of revetment to prevent meandering of the LMR. Through natural meandering, new backwater habitats are produced over time. However, the LMR is controlled to such an extent that few new backwaters are formed. Current backwater systems are predictably filling in with river sediments and it is vital to understand the importance of this heterogeneous environment before what remains is lost.

The LMR main channel includes semi-permanent "slackwater" patches (areas of reduced flow within or adjacent to the main channel) that have been shown to be important as in-stream refuges for biotic communities (Thorp et al. 1994; Reckendorfer et al. 1999; Thorp and Mantovani 2005; Deksne and Skute 2011). These patches consist of natural areas such as horseshoe islands (islands with a downstream opening to a slackwater pool), sandbar embayments, and secondary channels. There are also manmade areas that have the same effect, such as behind dikes and weirs. Regardless of being manmade or natural, these distinct, relatively quiescent habitats can serve as refuges from the fast, turbulent current of the main channel, and thus may be vital in the life-cycle of some large river biota (Sabol et al. 1984; Thorp et al. 1994; Reckendorfer et al. 1999; Thorp and Mantovani 2005; Deksne and Skute 2011).

1.5 Significance

This study was designed to test the importance of hydrologic connectivity for zooplankton population dynamics, use of an alternate identification technique, spatial distribution of haplotypes patterns, and cryptic species detection across the main channel and floodplain of the LMR. Zooplankton are a significant part of the riverine ecosystem, are an essential step in the food chain from phytoplankton to many fish, and are the first food source of many larval fish. The results of this study will substantially enrich understanding of the factors important in structuring zooplankton assemblages of the LMR by concentrating on questions of zooplankton assemblage structure across the variably connected floodplain system.

This is valuable research partly because little to no work has been done on zooplankton of the LMR, the largest (in terms of watershed area and discharge) river system in North America. Although there has been more work in recent years on river zooplankton, the complex combination of influences on assemblage structure remains incomplete. Large lowland river systems have multiple interconnected water bodies with varying degrees of hydrologic and nutrient exchange with the river. Even large backwater lakes have spatial variability and it may be too reductive to view them as a single habitat. The research here elucidates the patterns and dynamics of shifting zooplankton assemblages across the LMR providing a basis for further research.

Identification of zooplankton morphologically is time consuming and requires a large amount of experience. Development of alternate methods of identification that are more easily accomplished would free time otherwise allocated to painstaking morphological methods. However, proper testing of alternative methods is necessarily before they can be widely applied

in research. The critical analysis of genetic metabarcoding identification presented here adds to the growing body of research showing the strong potential for use in zooplankton studies.

Finally, although the influence of hydrologic connectivity on zooplankton assemblages has recently been examined in several rivers worldwide, little attention has been given to within species distribution patterns. Both haplotype distribution of three zooplankton species and cryptic species presence are presented here. The results of this portion of the study indicate that some species of zooplankton have haplotypes that are restricted even when the species as a whole is found across the landscape. Greater intraspecies variation was also found for *Brachionus calyciflorus*, indicating that there are multiple cryptic species residing in the study area. This study of zooplankton across a spatial and temporal gradient in the existing LMR floodplain will contribute to the understanding of the functional processes that govern the composition and regeneration of those assemblages and will give insight to similar systems in other areas of the world.

CHAPTER II: HYRDOLOGIC CONNECTIVITY AS A DRIVER OF TEMPORAL AND SPATIAL VARIATION IN ZOOPLANKTON ASSEMBLAGE STRUCTURE ACROSS A LARGE RIVER FLOODPLAIN

Abstract

Lateral hydrologic connectivity between the main channel of a river and its floodplain is seasonally and spatially variable in degree and duration. The pattern of connection influences the physical and chemical make-up of floodplain habitats, which I hypothesized should in turn lead to variation in the composition of aquatic communities along a connectivity gradient. To assess this hypothesis, I evaluated rotifer and crustacean zooplankton assemblage structure in floodplain lakes across a gradient of connectivity with the Lower Mississippi River. Between May 2015 and October 2016, 19 sites representing the connectivity gradient were sampled on multiple occasions for zooplankton and environmental variables. I analyzed assemblage structure as differences in taxa dominance (abundance changes, based on Bray-Curtis dissimilarities) and taxa turnover (presence/absence, based on βsim metric), across four broad connectivity categories (Ward and Stanford 1995) and across continuous environmental variables that distinguished these categories. For rotifers, dissimilarity in both taxa dominance and turnover were greatest between the most connected (eupotamal) compared to less

connected (para-, plesio-, and paleopotamal) floodplain lakes. However, within a connectivity classification, there were substantial differences in assemblage structure across the entire sampling period correlated with seasonal changes. For crustaceans, dominance was more variable seasonally than across the connectivity gradient, while turnover showed little association with either connectivity or seasonality. Highest abundances and taxa richness of both rotifers and crustaceans were in least connected lakes. The categorical classification generally worked well in discriminating environmental variables and zooplankton assemblages across connectivity among whole lakes, but did not recognize spatial variation in assemblage structure and environmental variables along the longitudinal axis of two large oxbow lakes. This within-lake connectivity gradient was not represented in a discrete, whole lake, classification system. This study highlights the importance of spatially and temporally variable connectivity for the structure and diversity of zooplankton assemblages in floodplain habitats. Some taxa were abundant and wide-spread across the gradient, while other taxa were uncommon and utilized a single connectivity category more than others. In this large, dynamic river system, maintenance of habitat diversity is especially critical for taxa restricted in habitat requirements.

2.1 Introduction

Landscape connectivity refers to the potential for movement or migration among distinct habitats of energy, water, other matter including nutrients and toxins, and organisms. Such movements may be passive, for example by diffusion or in the flow of the environmental medium, or active, by purposeful migration of animals from place to place. In aquatic systems, the degree and patterns of connectivity among discrete sites is a driving force in the spatial and

temporal structuring of biotic communities and ecological processes (Tockner et al. 1999; Ward and Tockner 2001; Lansac-Tôha et al. 2009).

The role of hydrologic connectivity to the main river channel is recognized globally in landscape patterns of biotic diversity and ecological processes (Junk et al. 1989; Pringle 2003, Harrison et al. 2017). With a flood pulse the river and floodplain merge, while at lower stage heights the floodplain disconnects from the river channel. This results in an ecologically complex system expanding and contracting in its aquatic and terrestrial components with the height of the river. However due to river channelization, levee construction, and floodplain loss, these systems are endangered world-wide. Floodplain rivers, if permanently cut off from their floodplains, are relegated to pipe-like conduits, sacrificing habitat and biotic diversity, productivity, and biogeochemical dynamics (Brooker 1985; Toth 1993; Elosegi and Sabater 2013).

The Lower Mississippi River (LMR) is a valuable setting to study the influence of connectivity on biotic assemblage structure across a large river floodplain. Although highly modified for flood control and navigation, the LMR remains free of impoundments and a wide range of aquatic habitats remains intact between the levees (maximum distance ~23 km, Biedenharn et al. 2018), having varying degrees of connectivity to the main channel (Baker et al. 1991; Pongruktham and Ochs 2015). Many of these backwaters are large oxbow lakes which may show a gradient in connectivity within a single lake due to their size and length. In support of navigation requirements, the hydrologic dynamics of the LMR are well-documented, but determination of the ecological dynamics of this system is minimal (Ochs et al. 2013), notably in organisms like zooplankton.

Zooplankton are an important group of organisms to study in river-floodplain ecosystems because of their critical role in food webs as grazers, predators, and prey. Further, while all zooplankton are motile, in a large river-floodplain systems maintaining a strong current, zooplankton are passengers of flow and thus highly influenced in their distribution across sites by hydrologic connectivity. Zooplankton differ by broad taxonomic category in traits affecting their spatial distribution across a connectivity gradient including their sizes, growth rates, swimming proficiency, mode of feeding, and adaptability to habitat conditions. Rotifers, for example, tend to be favored in high flow conditions because they are less impacted by suspended sediments (Kirk 1991) while having higher growth rates than crustacean zooplankton. However, when flow is reduced and suspended sediments drop out of the water column, the competitive efficiency and predation impact of crustacean zooplankton on rotifers can shift the assemblage structure (Thorp and Mantovani 2005). These observations have two implications. First, with high connectivity, both environmental variables and zooplankton assemblages homogenize across the system (Reckendorfer et al. 1999; Baranyi et al. 2002; Bozelli et al. 2015). As the river recedes and habitat heterogeneity develops upon disconnection of backwaters from each other and the main channel, there is opportunity for adaptive development in spatial variation of zooplankton assemblage structure (Picard and Lair 2005; Burdis and Hoxmeier 2011; Balkić et al. 2018). Second, across a river floodplain, a corresponding gradient from high to low is expected in the percentage of rotifers to crustaceans with a decrease in connection to the main channel (Kirk and Gilbert 1990; Basu and Pick 1996; Zimmermann-Timm et al. 2007).

Prior to the current study, the composition and dynamics of plankton assemblages across the full range of variably connected floodplain habitats of the LMR, and the relationship of

assemblage structure to connectivity, have not been examined. The only other study of zooplankton dynamics in this large river system was published several decades ago in an internal document of the U.S. Army Corps of Engineers (Sabol et al. 1984), and included samples collected only from the main channel, side channels, and a single backwater.

The primary objective of this study was to determine the relationship of hydrologic connectivity with the river main channel to zooplankton assemblage structure across the LMR floodplain. This study is important for what it reveals about the understudied LMR and as a comparison with other studies, generally conducted in smaller less-turbid rivers, of the role of hydrologic connectivity in structuring zooplankton community structure across the floodplain. To categorize connectivity state I investigated the applicability of the potamal classification categories of Ward and Stanford (1995). This classification system has recently been implemented in research investigating spatial patterns in zooplankton assemblages across connectivity (Goździejewska et al. 2016, Balkić et al. 2018). However, this system was developed on smaller rivers in Europe and hasn't been implemented in a river the scale of the LMR. Our research hypotheses were (1) Potamal states can be discriminated in the LMR floodplain by environmental variables related to hydrologic connectivity with the river; (2) large meander lakes exhibit a gradient of connectivity along their length that is reflected in both environmental variables and zooplankton assemblage composition; (3) Zooplankton assemblage structure in the LMR floodplain aligns with potamal state and environmental variables most closely associated with connectivity.

2.2 Methods

2.2.1 Sample sites

Nineteen sites were sampled on the LMR and its floodplain between river km 1031 and 998 (Table 2.1). Ten sites, chosen to represent varying degrees of connectivity, were each sampled eleven times between May 2015 and Oct 2016. An additional nine sites across the connectivity gradient were sampled once in May 2015, and four times between May and Oct 2016. Site connectivity was classified as eupotamal, parapotamal, plesiopotamal, or paleopotamal based on descriptions by Ward and Stanford (1995) (Figure 2.1). "Eupotamal" refers to the main channel and side or secondary channels connected at both ends to the main channel during average low water periods. "Parapotamal" refers to lakes that maintain a downstream connection with the river during typical annual low water stages. "Plesiopotamal" refers to lakes that connect during average yearly high river stage, but not at lower water stages. "Paleopotamal" refers to lakes that do not connect during average annual high water stages. As this classification indicates, connectivity at a particular site has a strong temporal, as well as spatial, component.

Names, connectivity level, and locations of sample sites are listed in Table 2.1, and shown in Figure 2.1. I determined minimum connectivity threshold necessary for a

Table 2.1. Sampling sites with location and lake surface area. Sites in bold were sampled 11 times throughout 2015 and 2016, remaining sites were sampled once in 2015 and 4 times between May 2016 and October 2016. Surface area measured using Google Earth at a stage height of 4.09 m at Helena, AR

Connectivity	Site	GPS Coordinates	Surface Area (km^2)
Eupotamal	**LMR Main Channel (LMR)**	34° 10.322' N 90° 54.278' W	-
	Island 63 Secondary Channel (I63)	34° 15.844' N 90° 44.835' W	1.42
	Island 64 Secondary Channel (I64)	34° 12.560' N 90° 52.106' W	1.44
	Sunflower Dike Field (SFD)	34° 10.563' N 90° 53.324' W	-
Parapotamal	**Upper Desoto Lake (UD - upstream)**	34° 10.785' N 90° 48.361' W	6.17
	Middle Desoto Lake-North (MDN - middle)	34° 08.626' N 90° 52.041' W	
	Middle Desoto Lake-South (MDS - middle)	34° 08.448' N 90° 52.129' W	
	Lower Desoto Lake-North (LDN - downstream)	34° 09.441' N 90° 53.273' W	
	Lower Desoto Lake-South (LDS - downstream)	34° 09.213' N 90° 53.476' W	
	Upper Mellwood Lake (UMW - upstream)	34° 14.790' N 90° 55.230' W	3.43
	Middle Mellwood Lake (MMW - middle)	34° 13.602' N 90° 56.198' W	
	Lower Mellwood Lake (LMW - downstream)	34° 11.464' N 90° 54.381' W	
Plesiopotamal	**Gloryhole Lake (GH)**	34° 14.633' N 90° 49.722' W	0.15
	Borrow Pit A (BPA)	34° 17.983' N 90° 49.026' W	0.16
	Borrow Pit B (BPB)	34° 17.857' N 90° 49.394' W	0.17
Paleopotamal	Graveyard Lake (GY)	34° 16.034' N 90° 47.421' W	0.02
	McWilliams Lake (McW)	34° 15.092' N 90° 48.138' W	0.28
	Old River Chute (ORC)	34° 15.745' N 90° 48.260' W	0.24
	Jim Samples Lake (JS)	34° 14.461' N 90° 48.794' W	0.08

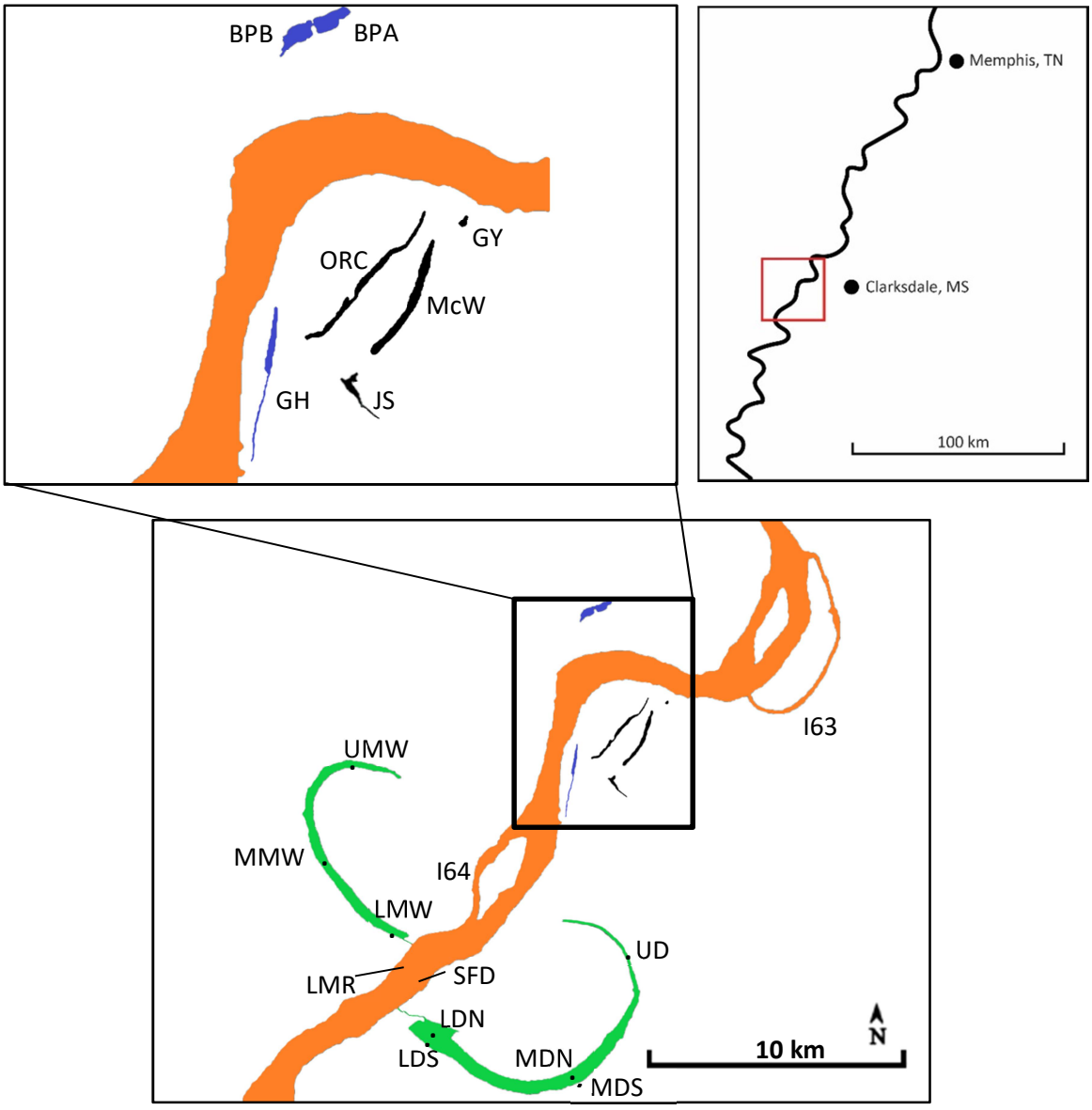

Figure 2.1. Map of LMR between river km 1031 and 998. Sample area in red box in top right. Orange = eupotamal sites; Green = parapotamal; Blue = plesiopotamal; Black = paleopotamal. Black dots in Mellwood and Desoto lakes indicate sample locations along lake axis. Abbreviations in Table 2.1.

site to connect to the main channel using remote sensing according to the method described in Oliver et al. (2016). Briefly, elevation of the sampling area was determined by aerial and bathymetry data. Connecting channels were identified between the main channel and floodplain study sites and highest elevation within a connecting channel was used as the connection threshold. I then categorized sites based on whether they would connect during the average high stage height from the previous 50 years and whether they disconnected during the average low stage height (Figure 2.2). For the years 2000-2016, the average percentage of time of connection by category was 98.6% (eupotamal excluding main channel), 82.9 % (parapotamal), 20.1 % (plesiopotamal), and 5.9 % (paleopotamal).

Eupotamal sites included the main LMR channel, two secondary channels behind Islands 63 and 64, and a dike field. Island 64 would lose upstream connection for a few days based on average low water (Figure 2.2), however I retained this secondary channel in the eupotamal category as the upstream disconnection would be on the order of days. Parapotamal sites included two long oxbow lakes formed by river "cutoff" excavations in 1942 by the United States Army Corps of Engineers - Memphis District (Mellwood Lake, 9.5 km length, and Desoto Lake, 12 km length). Plesiopotamal sites included three smaller lakes, one an abandoned main channel lake (Gloryhole), and two excavated borrow pits created during levee construction and located on the opposite side of the river. I was unable to sample Gloryhole during the Feb. and March 2016 sample dates as the high river stage in Jan. 2016 blocked access. Finally, paleopotamal sites included four smaller lakes, one formed as a scour hole during flooding (Graveyard), two abandoned main channel lakes (McWilliams and Old River Chute), and a small impounded lake (Jim Samples). For all lakes, samples were collected from a boat. For the two

large oxbows, samples were taken at multiple locations (three locations in Mellwood, five locations in Desoto). Otherwise, there was only a single mid-lake sample site (Figure 2.1).

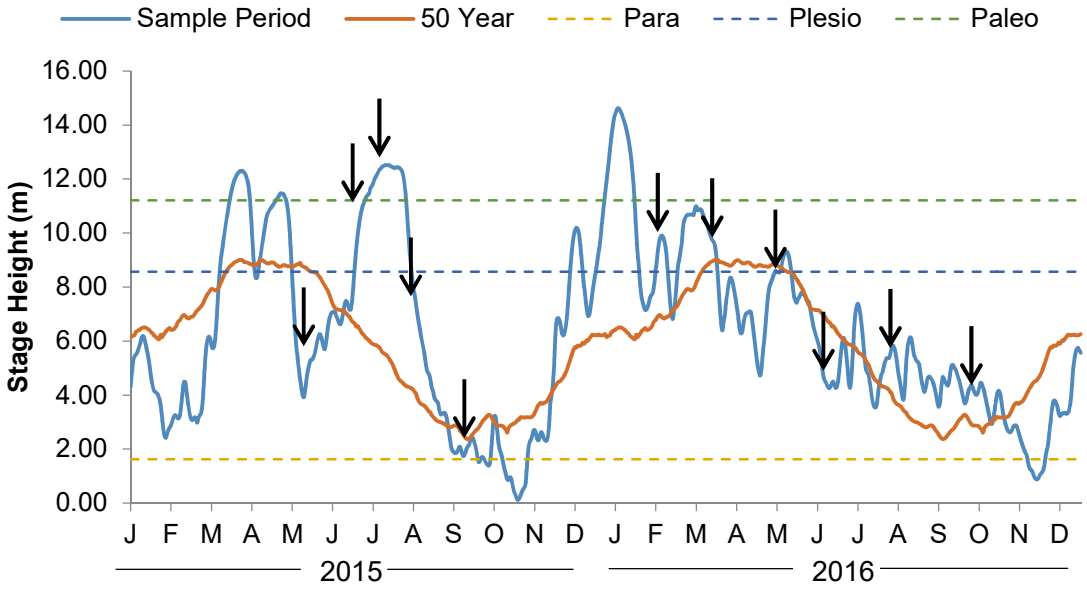

Figure 2.2. Stage height of LMR during the sampling period and 50 year average. Sampling dates as arrows. Connectivity category average connection threshold as dashed lines. Eupotamal not included as they would maintain connection throughout the sampling period. All measurements in reference to river gage at Helena, AR, maintained by the United States Army Corps of Engineers – Memphis district.

2.2.2 Water sampling and filtration

Triplicate water samples were collected from each sample site on each sample date. Grab samples were collected at 0.5 m below the surface using 250-mL Nalgene bottles and stored in a cooler with ice for transport back to the lab. In the lab, 100 mL of each replicate was filtered through 47-mm diameter Whatman GF/F filters, and stored in aluminum foil at -60°C, for subsequent chlorophyll analysis. The filtrate was stored frozen in 500-mL Fisherbrand sterile sampling bags for dissolved chemistry analysis. Twenty-five mL of each replicate was filtered

through 25-mm diameter Whatman GF/F filters, and stored as above, for analysis of particulate nitrogen and carbon.

Surface water temperature (Temp), dissolved oxygen (DO), pH, depth, and GPS coordinates were taken at each site on each sample date. Stage height was obtained from the Helena, AR, river gage maintained by the United States Army Corps of Engineers – Memphis district. Chlorophyll-a (Chla) levels were measured in the lab by spectrometry (Wetzel and Likens 2000). Turbidity (Turb) was measured using a Hach model 2100A turbidimeter. Particulate organic carbon (POC) and particulate nitrogen (PN) were analyzed at either the University of California Davis using an Elementar Micro Cube elemental analyzer interfaced to an Isoprime VisION IRMS, or the University of Southern Mississippi using a Costech ECS 4010 elemental analyzer. Total dissolved organic carbon (TDOC) and total dissolved nitrogen (TDN) were measured at the University of Mississippi using a Shimadzu TOC-L CSH.

2.2.3 Zooplankton sampling

Crustacean and rotifer zooplankton were sampled separately at each site. Crustacean samples were collected by vertical tows of a 153-µm mesh plankton net. The volume of water sampled was calculated based on the total length of tows (5 tows of 5-m length per sample) and the diameter of the opening of the plankton net (12-cm) to equal 283 L per sample. Vertical tows are impossible in the high flow of the main channel of the LMR, but the LMR is continuously well-mixed and unstratified from top to bottom (Ochs et al. 2010). Thus, from this site, 80 L of near surface water was poured through the plankton net. Rotifer samples were collected at all sites by submerging a 1-L Nalgene bottle to 0.5 m under the surface and allowing it to fill there.

The water was then passed through a 32-µm mesh sieve for concentration; this process was repeated to obtain a composite sample from 8 L.

Concentrated preserved samples were stored in 50-mL centrifuge vials using 95% ethanol for a final concentration of 70% ethanol. Preserved zooplankton sample abundance and identification were determined by light microscopy (Olympus IX70 inverted and AX70 compound at 40x-600x magnification; Olympus SZX12 dissecting at 50x magnification). Juvenile crustacean stages were not identified but were included in total zooplankton abundances for each site. Preserved sample volume was normalized to 50 mL in the lab for convenient subsampling and stored at 0°C. Rotifer samples were subsampled and counted using a 1-mL Sedgewick-Rafter chamber. Crustacean samples were subsampled and counted using a Ward counting wheel with individuals transferred to microscope slides for finer taxonomic identification. Additionally, live (unpreserved) samples were collected and transported in 250-mL Nalgene bottles to aid in identification and enumeration. In the majority of cases, live and preserved samples were identified to at least genus. Exceptions include the copepod order Harpacticoida, cladoceran family Macrothricidae, rotiferan class Bdelloidea, and for a few difficult to identify individuals, the crustacean orders Cyclopoida and Calanoida. Aside from Macrothricidae, these broad taxonomies combined represent less than 1% of total individuals. I present richness here as all taxa identified, regardless of taxonomic level. Rotifers were identified according to Koste (1978), Stemberger (1979), Koste and Shiel (1987), Shiel (1995), Nogrady and Segers (2002), and Wallace and Snell (2010). Crustaceans were identified according to Dodson et al. (2010) and Reid and Williamson (2010).

2.2.4 Data analysis

Data were visualized and analyzed using the vegan (Oksanen et al. 2018), ggplot2 (Wickham 2016), randomForest (Liaw and Wiener 2002), varSelRF (Diaz-Uriarte and Alvarez de Andres 2006; Diaz-Uriarte 2007), GGally (Schloerke et al. 2016), lme4 (Bates et al. 2015), and emmeans (Lenth 2018) packages in R 3.5.1 (R Core Team 2018). Random number generator seeds were set to "5" for all analyses to ensure reproducibility. All environmental data were natural log-transformed before analyses to meet assumptions of parametric statistical tests. Correlation of environmental variables was tested using the GGally package.

To test the applicability of the Ward and Stanford (1995) classification, the randomForest machine learning package was used to determine which continuous environmental variables were important in classifying connectivity categories and which samples were misclassified in the constructed model. Variable importance is ranked from high to low by randomForest based on the mean decrease in classification accuracy when values of a variable are randomly permuted in a node of a tree; this process was repeated for all trees (Breiman 2001). Those variables with a high mean decrease in accuracy when randomly permuted are considered the most important variables for classification. The varSelRF package was used to remove the least important variables until classification accuracy drops (first major breakpoint in mean decrease in accuracy, Appendix C); those variables remaining are considered the most important in distinguishing connectivity classifications. Variables found to be the most important were analyzed with linear mixed-effects models and pairwise least squares means comparisons (Kenward-Roger df, Tukey HSD), with sampling date added to the model as a random effect, to determine significant differences among connectivity classifications using the lmer and lsmeans

functions of the lme4 and emmeans packages, respectively. I also analyzed differences in alpha diversity (measured as taxa richness) and abundance across connectivity in the same manner as environmental variables. Further, I analyzed important environmental variables, richness, and abundance across the longitudinal sampling axis of the two large parapotamal lakes (connectivity declining from downstream to upstream) along with plesiopotamal lakes in order to determine potential connectivity states within large backwaters.

Rotifer and crustacean samples were analyzed separately to avoid one group driving patterns over the other. The main channel site was excluded from analyses of crustacean samples due to low sample size. The June 2015 samples of paleopotamal sites were lost during transfer and are absent from analyses. To test our hypothesis that zooplankton assemblages align with potamal state, I first quantified beta diversity, using two distinct metrics, the Bray-Curtis dissimilarity index and βsim (Koleff et al. 2003; Barwell et al. 2015). Bray-Curtis compares abundances of taxa among samples to investigate differences in dominance, or shifts in the most abundant taxa. βsim (Lennon et al. 2002 based on Simpson 1943) compares turnover of taxa, or shifts in the presence/absence of taxa, giving equal weight to rare and abundant taxa. To visualize patterns in beta diversity, dissimilarity matrices were ordinated by nonmetric multidimensional scaling (NMDS) (Kruskal 1964) with important environmental variables included as vectors. The combination of environmental variables that rank-order correlated most with each dissimilarity matrix was determined using the bioenv function in the vegan package and those variables were plotted as vectors on ordinations. The randomForest and varSelRF packages were used to determine which taxa were driving differences in assemblage structure across connectivity. The results of this process match more closely with those of the Bray-Curtis

NMDS analysis, rather than βsim NMDS, as abundances of zooplankton taxa are used instead of presence/absence.

2.3 Results

2.3.1 Physical/chemical characteristics

The hydrograph generally followed the normal historical trend of high water in spring and decreasing through the fall (Figure 2.2). However, the highest river elevations in 2015 were in summer (July), and in 2016 in winter (January). In 2015 stage height varied at a relatively low frequency but of high amplitude (> 8-m between adjacent peaks and troughs), while in 2016 there was a high frequency of variation in stage height but of lesser amplitude (< 4-m). Environmental characteristics varied with connectivity in the investigated section of the LMR (Table 2.2, Appendix A).

In general, environmental characteristics excluding temperature varied as much or to a greater degree among sites concurrently than they did within sites seasonally (Appendix A). For instance, the range in values of TDN across sites on a particular sample date was similar to the range in values across sites on all sample dates. In contrast, temperature varied little among sites on a particular sample date, but substantially with time for all sites.

There was only one instance of strong pairwise correlation among environmental variables, between POC and PN ($r = 0.93$), which caused us to remove PN from subsequent analyses (Appendix B). The remaining variables were used in a random forest machine learning classification for connectivity. The model correctly classified 87.5% of the samples into connectivity categories which supports the hypothesis that these categories can be distinguished

by environmental variables in the LMR. Mis-classified samples were primarily from plesiopotamal and paleopotamal.

Table 2.2. Environmental variables grouped by connectivity classification. Values shown are the range between the means of minimum and maximum values across the entire sampling period (May 2015 to October 2016).

Connectivity	Temp (°C)	DO (mg/L)	pH	Depth (m)	Turb (NTU)	Chla (µg/L)	POC (mg/L)	PN (mg/L)	TDOC (mg/L)	TDN (mg/L)
Eupotamal	5.7	5.3	7.17	5.6	25.47	2.49	1.49	0.20	2.66	1.09
	30.3	16.1	8.05	20.0	73.83	16.72	6.44	0.71	12.00	2.46
Parapotamal	7.1	5.4	7.51	3.1	4.11	14.97	0.78	0.18	3.73	0.30
	31.7	15.6	8.56	11.3	22.67	66.85	4.09	0.70	11.82	1.73
Plesiopotamal	22.5	3.4	7.51	0.8	8.27	8.42	1.26	0.23	4.38	0.24
	32.8	10.9	9.28	5.8	29.33	140.64	11.22	1.78	14.69	1.52
Paleopotamal	8.5	5.1	7.63	2.9	2.78	10.50	0.87	0.14	3.41	0.17
	32.8	18.7	8.52	4.3	11.25	45.22	4.40	0.72	12.64	0.90

The most important set of variables for classifying connectivity identified using randomForest and varSelRF, from greatest to lesser importance, were TDN, Turb, Chla, Depth, and DO (Appendix C). These five variables, along with Temp, were retained for subsequent analysis of connectivity classification and the principal drivers of variation in zooplankton assemblage structure. Temp was retained because, although it isn't an important classifier of connectivity, it is associated with, and considered a surrogate for, seasonality/sample date.

Among connectivity categories, there were significant differences in environmental parameters (Figure 2.3). TDN decreased and Temp increased slightly with decreasing connectivity. Eupotamal sites were consistently highest in turbidity and an order of magnitude lower in Chla than more disconnected sites (Table 2.2). Parapotamal sites exhibited the highest levels of DO across connectivity. Plesiopotamal sites were highest in Chla, lowest in Depth, and

intermediate in Turb between eupotamal and parapotamal sites. Paleopotamal sites were intermediate or similar in Chla and DO between eupotamal and the other two connectivity classifications.

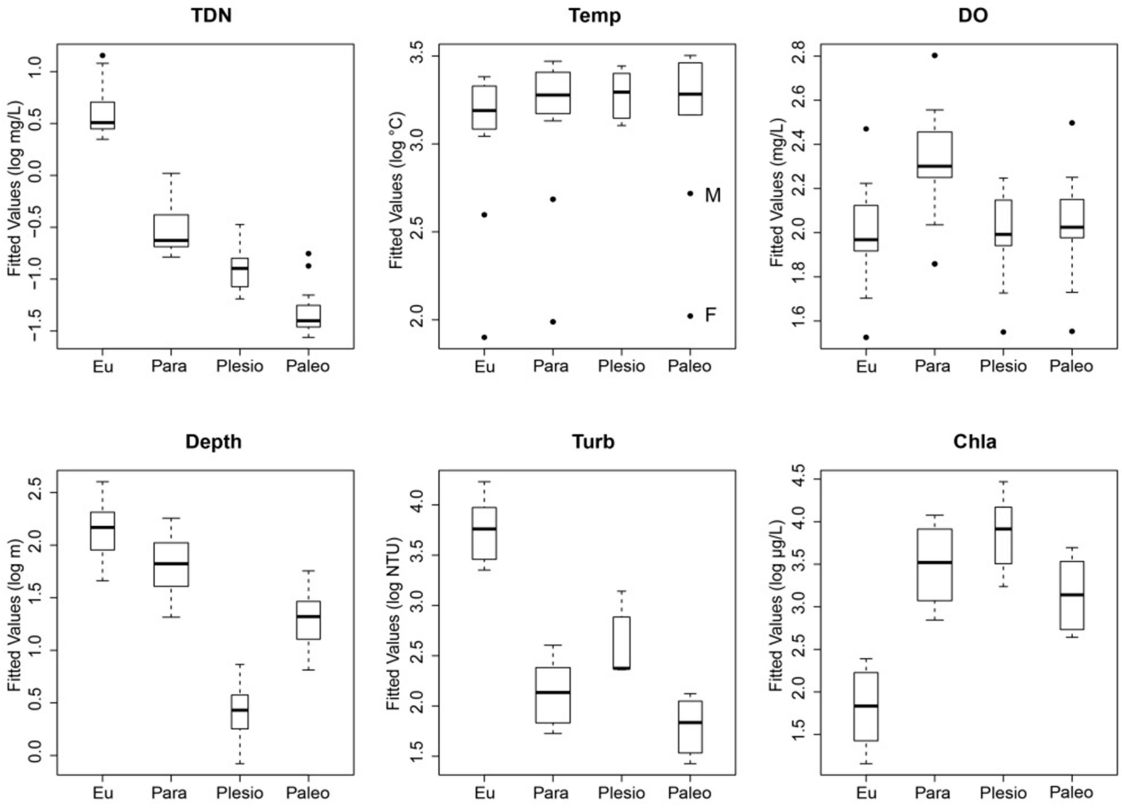

Figure 2.3. Fitted values of environmental variables by connectivity. Date included as a random effect. Dark lines are medians, boxes are inter-quartile range, whiskers are for data within 3X inter-quartile range, and points are outliers. Outliers in the temperature plot are for samples collected in February (F) and March (M).

In the spatial analysis of the two large oxbow lakes, TDN and Turb both decreased from downstream (closer to the connecting channel to the river) to upstream areas (Figure 2.4). Depth was greatest in middle areas. Chla increased from downstream to upstream and was significantly higher upstream compared to downstream (t-ratio = -2.988, P = 0.02). These results indicate that

large oxbows have a gradient along their length in environmental variables associated with connectivity. However, neither DO nor Temp varied significantly along the lake axes.

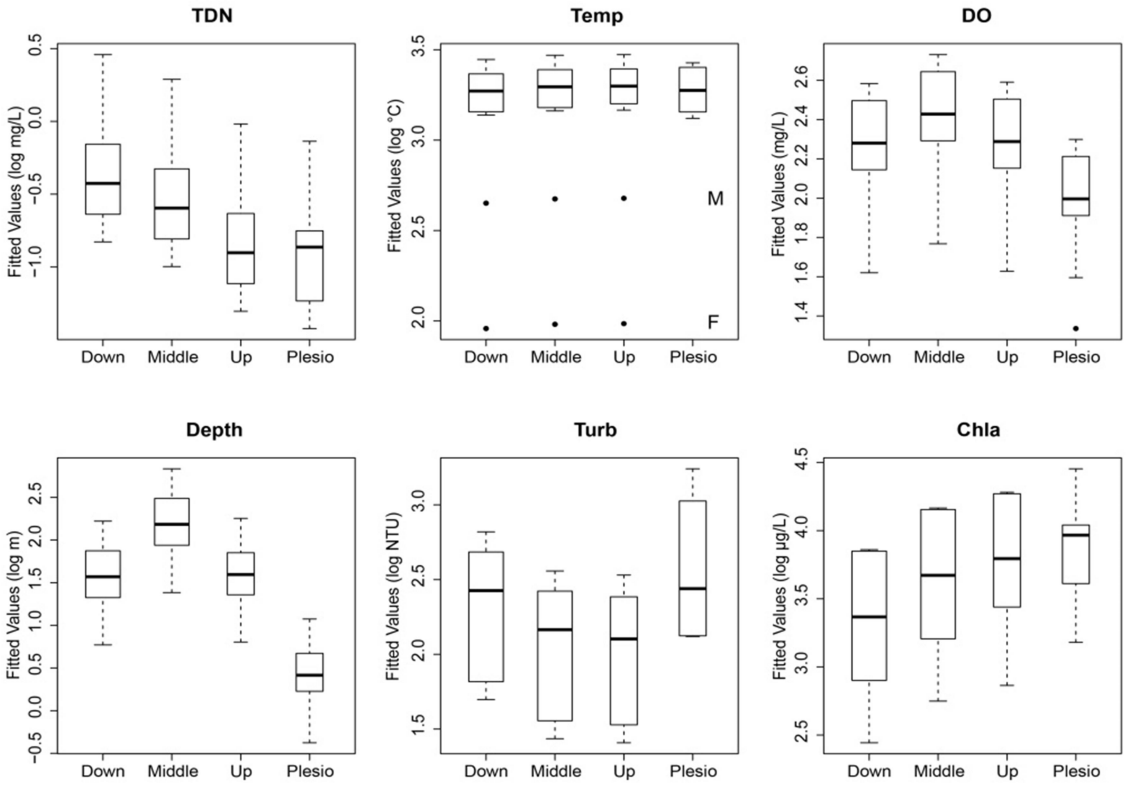

Figure 2.4. Fitted values of environmental variable across large parapotamal oxbow lakes (Mellwood and Desoto). Plesiopotamal included for comparison. Date included as a random effect. Down refers to downstream area (higher connectivity); up refers to upstream area (lower connectivity). Dark lines are medians, boxes are inter-quartile range, whiskers are for data within 3X inter-quartile range, and points are outliers. Outliers in the temperature plot are for samples collected in February (F) and March (M).

2.3.2 Zooplankton assemblages

Throughout the sample area, 70 distinct taxa were identified. Of these, 41 were rotifers identified to class (1), genus (7), and species (33), and 29 were crustaceans identified to order (3), family (1), genus (16), and species (9). Rotifer richness and abundance both increased with decreasing connectivity (Figure 2.5). Crustacean richness and abundance followed similar

patterns of highest in paleopotamal and lowest in eupotamal and plesiopotamal. Eupotamal sites were not significantly different compared

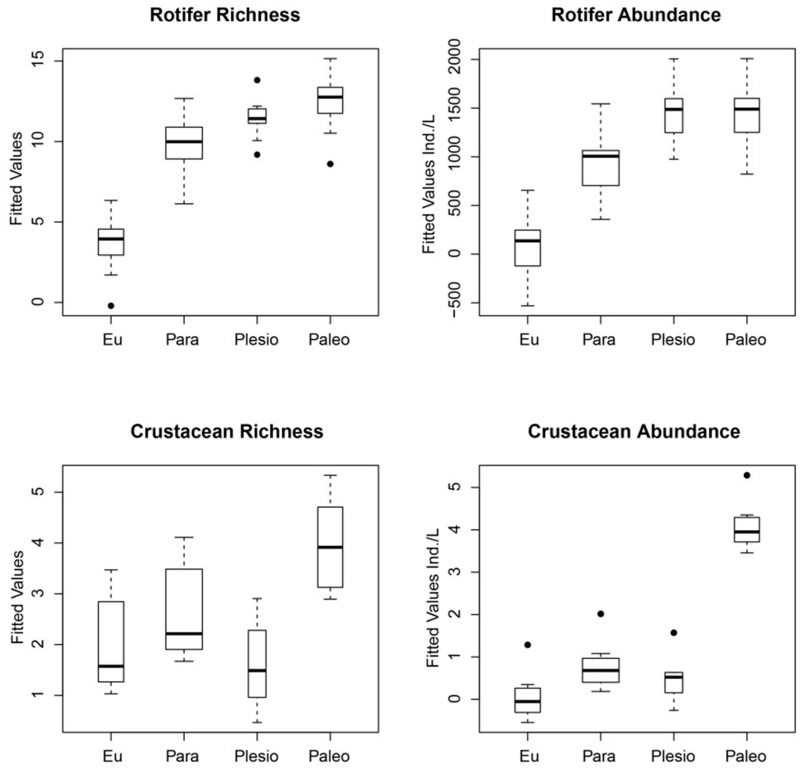

Figure 2.5. Fitted values of richness and abundance of rotifers and crustaceans across connectivity. Date included as a random effect. Dark lines are medians, boxes are inter-quartile range, whiskers are for data within 3X inter-quartile range, and points are outliers.

to parapotamal or plesiopotamal across crustacean measures (Table 2.3). Further, richness and abundance were not significantly different across the two large lakes for rotifers and crustaceans.

Richness and abundance for both rotifers and crustaceans was highest on average in spring (May and June) and lowest in winter (February). Rotifers made up 97-99% of the total zooplankton abundance across all samples. Abundances of rotifers ranged from 3.13 L-1 to 5700 L-1 per sample. Eupotamal samples were dominated by *Keratella cochlearis* (Figure 2.6A)

Table 2.3. P-values from linear mixed effects models and pairwise least squares means comparisons (Kenward-Roger df, Tukey HSD) of richness (white) and abundance (grey) for rotifers and crustaceans.

Rotifers	**Eu**	**Para**	**Plesio**	**Paleo**
Eu	-	<0.0001	<0.0001	<0.0001
Para	<0.0001	-	0.27	<0.0001
Plesio	<0.0001	0.04	-	0.22
Paleo	<0.0001	0.003	1	-
Crustaceans	**Eu**	**Para**	**Plesio**	**Paleo**
Eu	-	0.15	0.56	<0.0001
Para	0.28	-	0.02	0.0006
Plesio	0.96	.84	-	<0.0001
Paleo	<0.0001	<0.0001	<0.0001	-

throughout the sampling period, and were restricted mainly to loricate rotifers from the Brachionidae family. Floodplain lakes were characterized by seasonal shifts in dominance between *Anuraeopsis navicula*, *Brachionus caudatus*, *Polyarthra remata*, *Polyarthra vulgaris*, and *Synchaeta spp*. (Figure 2.6A). However, high abundance (>200 L-1) of *Asplanchna priodonta* were found in mid-spring of both years (May) in floodplain lakes, and *Trichocerca pusilla* maintained a near-continuous, albeit sometimes low abundance, presence throughout the sampling period. Also, *Brachionus angularis* and *Filinia sp.* were both abundant in late summer but not in numbers high enough to surpass the five aforementioned dominant species of floodplain lakes. Crustacean abundances ranged from 0.02 L-1 to 17.86 L-1 per sample. Eupotamal samples had very few crustaceans in general (< 0.32 L-1) and mainly consisted of *Bosmina longirostris* and *Acanthocyclops spp.* (Figure 2.6B). Sites within connectivity categories other than eupotamal were dominated by the cladocerans *Diaphanosoma spp.*

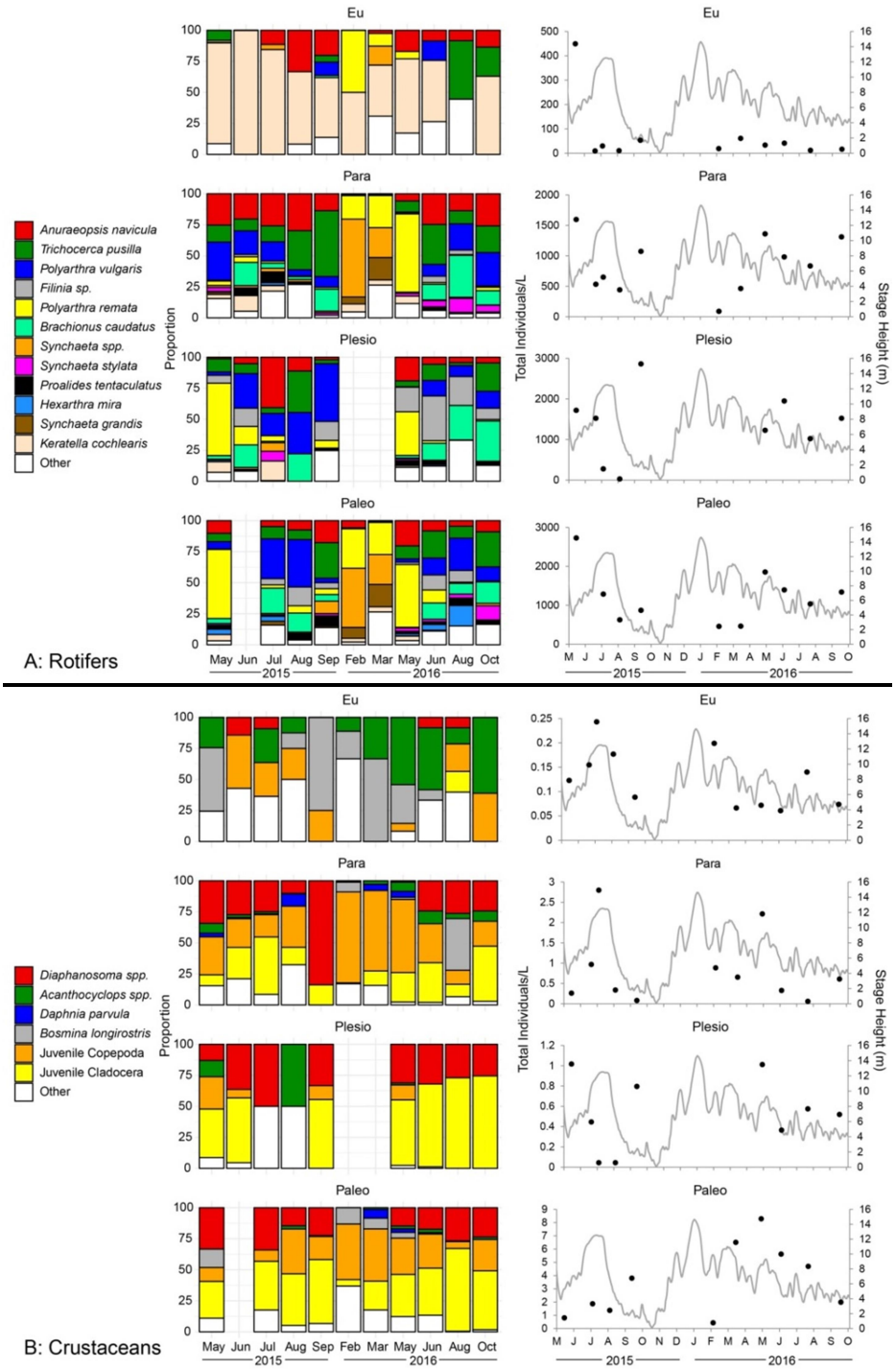

Figure 2.6. Proportional changes over time in important zooplankton taxa for distinguishing connectivity in the LMR . Taxa listed by importance as indicated from machine learning analysis. *Keratella cochlearis* included because of high proportion in eupotamal sites and juvenile crustaceans included because of high proportion across sites. Plots on right show average total zooplankton abundances by connectivity level, stage height included as grey line for reference.

and *Daphnia parvula*. In these sites, *Ceriodaphnia dubia*, *Moina micrura*, and the invasive species *Daphnia lumholtzi* were occasionally present in high abundances.

Figure 2.6 summarizes changes in taxa distinguishing connectivity category over the sampling period selected using the varSelRF package. Eleven rotifer and four crustacean taxa were selected as the most important in distinguishing connectivity. Although *Keratella cochlearis* was not considered important in this sense, I included it in the figure because it was consistently a high proportion of the total abundance of zooplankton in eupotamal sites. I also included juvenile crustaceans as they were the largest proportion of total abundance across all connectivities. The most important single rotifer taxon for distinguishing connectivity category was: eupotamal - *Anuraeopsis navicula*, parapotamal – *Anuraeopsis navicula*, plesiopotamal – *Filinia sp.*, and paleopotamal – *Hexarthra mira* (Figure 2.6A). For crustaceans, the most important taxon for classifying connectivity category was *Diaphanosoma spp.* (Figure 2.6B).

Rotifer abundance in the river main channel and other eupotamal sites was very high in May 2015, after which abundances were consistently below 100 L-1 (Figure 2.6A). In the floodplain lakes, rotifer abundance was highest in spring of each year and steadily reduced through fall. This pattern is most clearly seen in paleopotamal sites. Crustacean abundance in eupotamal sites was highest in July 2015 and February 2016. For parapotamal sites, crustacean abundance was highest in July 2015 and May 2016. Plesiopotamal sites had highest crustacean abundance in May of each year. Finally, paleopotamal sites had highest crustacean abundance in May 2016.

NMDS ordinations summarize differences in beta diversity between samples with vectors showing increases in the given environmental variable across ordination space (Figure 2.7). A

combination of TDN, Turb, Temp and Chla had the highest correlation (r=0.68) to the rotifer Bray-Curtis dissimilarity matrix (dominance differences) in the bioenv analysis. Turb, Chla, Temp, and DO had the highest correlation (r=0.36) to the crustacean Bray-Curtis dissimilarity. Chla and Temp had the highest correlation (r=0.40) with the rotifer βsim dissimilarity matrix (turnover). TDN, Temp, Turb, and DO had the highest correlation (0.37) with crustacean βsim dissimilarity. However, a strong temporal pattern is evidenced by the inclusion of Temp within each analysis. There was clear separation in rotifer dominance between eupotamal and all other connectivity categories along the Turb, TDN, and Chla vectors and separation seasonally along the Temp vector, with the largest dissimilarities occurring across connectivity (Figure 2.7A). For crustacean dominance (Figure 2.7B), eupotamal, plesiopotamal, and paleopotamal sites grouped with other samples of the same category. Parapotamal samples were more dispersed in ordination space, but generally fell between eupotamal and other categories. However, unlike the rotifers, the largest dissimilarities are within groups across seasons. Separation of groups was less clear in the turnover ordinations. However, for rotifers there was some visible separation between eupotamal vs. plesiopotamal and paleopotamal sites (Figure 2.7C). Crustacean turnover (Figure 2.7D) shows the least separation of samples with a few small groupings in an otherwise mixed ordination.

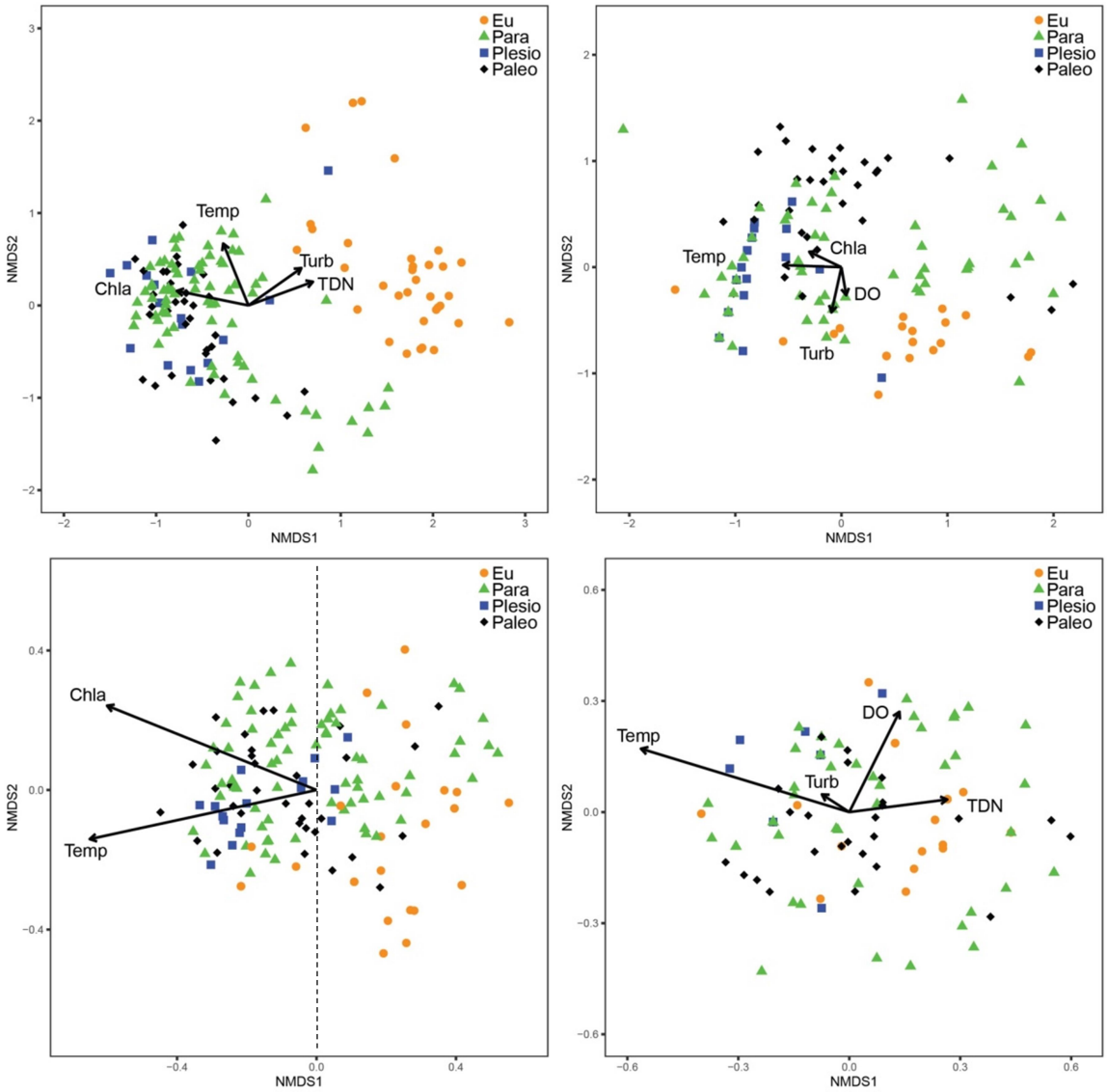

Figure 2.7. NMDS ordinations of Bray-Curtis dissimilarity representing differences in taxa dominance (A and B) and βsim representing turnover of taxa (C and D). Dashed line in C indicates pronounced separation of eupotamal vs. plesiopotamal and paleopotamal sites. Vectors included in each ordination were selected based on bioenv highest correlation results.

2.4 Discussion

The results presented here are mostly supportive of the original hypotheses, namely that connectivity categories can be distinguished by environmental variables in large rivers such as the LMR, a connectivity gradient exists across large floodplain lakes, and zooplankton assemblages align with connectivity categories and environmental variables.

The classification system of Ward and Stanford (1995) generally was accurate in distinguishing among potamal categories. However, the LMR is confined and levee to levee inundation occurs during most years. Thus, the LMR may not contain all true connectivity types, most notably paleopotamal sites. Nevertheless, the majority of samples were classified into their pre-determined categories by machine learning regardless of using environmental variables or rotifer/crustacean taxa data. The mismatch results of machine learning were mainly in the paleopotamal and parapotamal samples. A portion of the mismatches of paleopotamal samples were from Graveyard Lake and misclassified as parapotamal. Scour holes, like Graveyard, tend to be deep, but a shallow depth was one of the more important variables in categorizing paleopotamal sites. Thus, perhaps samples from Graveyard were mismatched into parapotamal in part because of the greater depth of the lake.

Desoto and Mellwood lakes are relatively large and long oxbows with a connecting tie-channel only at the downstream end of the lakes for much of each year. Upstream areas of both lakes had lower TDN and Turb, higher Chla, and intermediate assemblage dissimilarities between the downstream areas and plesiopotamal classifications. These results suggest a connectivity state in between the rest of the parapotamal sites and plesiopotamal classifications, one that has continual hydrologic connectivity but receives that connection over a long distance

and through a large volume of water from their downstream connection, for much of the year. This indicates that a connectivity gradient is present in large floodplain lakes. However, there weren't significant differences in abundance and richness of zooplankton and assemblage dissimilarities were relatively small between upstream areas and middle/downstream samples areas compared to upstream and plesiopotamal samples during low stage heights. These results suggest that zooplankton assemblages were unaffected by the environmental gradients that were observed across these large lakes. However, during high river stages (>10 m), zooplankton assemblages in upstream areas of large lakes were less affected by connectivity than plesiopotamal sites. This pattern is exemplified by plesiopotamal samples being more similar to eupotamal than are parapotamal (Figure 2.7A and B). This is likely caused by smaller lakes being completely washed-out while zooplankton within the greater volume and connection distance of upstream large lakes are protected. In other words, the effects of connectivity are a function of lake size, being more pronounced in small volume lakes than in larger lakes.

As expected, rotifer abundances were reduced during and shortly after connection events followed by a jump in abundance and steady decrease there after (Figure 2.6A). These results follow those of Baranyi et al. (2002) who found that during periods of high connectivity, abiotic and wash-out effects control zooplankton assemblages and after disconnection fast growing rotifers dominate but are later replaced by more competitive and predaceous crustacean taxa. Crustacean abundances had a dissimilar pattern to rotifer abundances in that they increased during high river stage in eupotamal and parapotamal sites (Figure 2.6B). These results would seem to be counter to those of Baranyi et al. (2002). It may be that increased crustacean abundances in eupotamal sites are due to wash-out from other sites. Górski et al. (2013) found

the highest numbers of crustaceans in flooded forests, which is the major terrestrial habitat in the LMR floodplain (Baker et al. 1991), and Flinn et al. (2005) found that dense vegetation increased zooplankton abundance. Considering the large expanse of terrestrial area inundated in the LMR during high stage height, perhaps high production of crustacean zooplankton combined with reduced predation by fish in inundated forest outweighs losses from wash-out in parapotamal sites.

Results from the correlation between beta diversity and environmental variables support the hypothesis that the structure of zooplankton assemblages are driven by lateral hydrologic connectivity but also have a seasonal component independent of connectivity. Temp was correlated to all zooplankton beta diversity differences supporting the seasonal component of assemblage differences, while TDN, Chla, Turb, and DO were included in different combinations representing connectivity differences in assemblage. Turb, a major indicator of connectivity in the LMR, was chosen in three out of four ordinations suggesting that suspended sediment plays a major role in zooplankton assemblages of the LMR. Although the Upper Mississippi and Ohio rivers contribute more than half of the water volume to the LMR, the majority of suspended sediments come from the Missouri River (Turner and Rabalais 2004). Our results for eupotamal sites closely resemble those from prairie rivers with high sediment load in the Missouri River basin, where there were low abundances of crustaceans and dominance of rotifers (Thorp and Mantovani 2005). In contrast, the other two major tributaries of the LMR, the Ohio and Upper Mississippi rivers, have substantially higher abundances of crustacean zooplankton in eupotamal sites (Thorp and Mantovani 2005; Burdis and Hoxmeier 2011). However, I cannot ignore the influence of dam structures along the Upper Mississippi and Ohio

rivers, which convert lotic rivers to more lentic conditions and in turn reduce turbidity. These comparisons give farther support to zooplankton assemblages being influenced by suspended sediment, which can negatively affect crustacean zooplankton (Kirk and Gilbert 1990; Kirk 1991; Arendt et al. 2011) and reduce light availability for zooplankton food sources such as phytoplankton (Ochs et al. 2013).

2.5 Conclusion

I have shown that spatial and temporal variation in hydrologic connection and habitat structure across the LMR floodplain drives spatial and temporal patterns in variation in structure of zooplankton assemblages. Historically, much of the emphasis on research of the LMR has been focused on the hydrology and maintenance of the main shipping channel with less attention given to floodplain water bodies and ecological function in general. Our results emphasize the need for variably-connected floodplain habitats for supporting biological diversity in large river systems. Although the most abundant taxa in this study usually occur across all connectivities of the LMR, less abundant taxa take advantage of an individual connectivity over others (Figure 2.6). Thus, it appears that, as for other organisms and ecosystems, habitat heterogeneity in the LMR floodplain is particularly critical for zooplankton taxa that are more specialized in their habits and restricted in where they can live. I suggest the maintenance of variable connectivity to floodplain lakes is crucial for maintaining zooplankton diversity in large rivers. Maintenance of current connecting channels between the floodplain and main channel will likely be needed in the future to prevent obstruction of connectivity by sediment deposition. Historically, the LMR would meander constantly creating and reshaping aquatic habitat, but with confinement by riprap, revetment and levees, the LMR is now mostly fixed in place. New aquatic habitat is less

likely to be formed, and current floodplain lakes are slowly filling with sediment (Hudson et al. 2008). Hence, in the future engineering may be needed to maintain or restore aquatic habitat heterogeneity in support of biodiversity of this large river-floodplain system.

CHAPTER III: COMPARISON OF DNA METABARCODING AND MORPHOLOGICAL IDENTIFICATION OF ZOOPLANKTON ASSEMBLAGES ACROSS A LARGE RIVER FLOODPLAIN

Abstract

Zooplankton are vital to the ecological function of aquatic habitats. Classic morphological methods for zooplankton identification are difficult and time consuming. Metabarcoding has shown promise in identifying zooplankton but has not been tested in riverine systems and across a hydrologic connectivity gradient. I assessed the performance of identification of zooplankton using metabarcoding and compared results to morphological identification. There were 82 taxa, at all levels, found using metabarcoding compared to only 43 found morphologically. Further, metabarcoding found a similar number of rotifer species-level assignments and more crustacean species compared to analysis by morphology. However, few species-level assignments were shared between the two methods. The most abundant taxa were the same across both methods; however, metabarcoding found hidden diversity in the form of multiple zero-radius operational taxonomic units (ZOTUs) assigned to the same species. Metabarcoding presented similar, although more defined, patterns in zooplankton beta

diversity across connectivity for both rotifer and crustacean genetic datasets compared to morphological datasets. Metabarcoding mainly suffered from issues related to reference databases and inability to quantify abundances. Until those issues are resolved it is recommended to continue to use morphological datasets in conjunction with metabarcoding for zooplankton studies.

3.1 Introduction

Zooplankton play an important role in aquatic ecosystems as grazers and predators of other plankton, prey to larger invertebrates and fish, in cycling nutrients between organic and inorganic forms (Vanni 2002), and are useful as bioindicators of environmental condition due to their strong response to abiotic and biotic controlling factors (Parmar et al. 2016). Precise determination of zooplankton assemblage composition depends on zooplankton identification to the lowest taxonomic level possible. Traditional morphological methods for identifying zooplankton are time-consuming and require a large amount of guidance and experience to accomplish. Morphological identification can also miss substantial hidden genetic diversity and potential cryptic species. Several widespread rotifer species are known to be cryptic species complexes (Ortells et al. 2003; Suatoni et al. 2006; Walsh et al. 2009; Stelzer et al. 2011; Xiang et al. 2011; Cieplinski et al. 2017). In the case of copepods, naupliar larvae cannot be identified using morphological methods, resulting in potential underestimation or omission of taxa from assessments of community composition.

An alternative approach for determination of zooplankton assemblage structure is by genetic analysis using DNA metabarcoding. Metabarcoding involves the extraction of DNA from bulk assemblage samples followed by PCR amplification of a given locus (e.g. mitochondrial

cytochrome oxidase subunit I). Sequences from amplified samples are then identified against a reference database. Advantages of this approach include the precision with which zooplankton can be identified and being able to process a larger sample in a fraction of the time compared to that required for morphological identification, providing a better opportunity to identify both rare and juvenile individuals. Recently, the use of metabarcoding has shown promise for identifying zooplankton assemblage diversity in freshwater and marine environments (Clarke et al. 2017, Yang et al. 2017).

Zooplankton ecology of freshwater systems has been studied more in lentic compared to lotic systems and, to our knowledge, no study has used metabarcoding to analyze zooplankton in relation to connectivity in a large river system, and to compare results with traditional morphological methods. In this study, I applied metabarcoding genetic techniques and morphological analysis across a gradient of hydrologic connectivity to the main channel in the floodplain of the Lower Mississippi River (LMR). My primary objective was to evaluate the relative merits of each approach for the analyses of zooplankton across the connectivity gradient. Specifically I asked: 1) Do patterns in zooplankton assemblages across the connectivity gradient derived from metabarcoding agree with morphological identification? 2) Are additional low abundance taxa that are absent from morphological data detectable using metabarcoding?

3.2 Methods

3.2.1 Study site – the Lower Mississippi River floodplain

This study was conducted in the floodplain of the Lower Mississippi River. The LMR, like many large rivers, has been heavily modified for flood control and navigation (Baker et al. 1991; Alexander et al. 2012). Despite these modifications, the LMR retains a wide range of

extant aquatic habitat within the levee system, including wetlands and lakes varying in timing and duration of connection with the main channel (Baker et al. 1991; Pongruktham and Ochs 2015). Due to variability in main channel connectivity, these floodplain habitats exhibit wide environmental gradients spatially and temporally (Chapter II).

3.2.2 Sample collection

Sample site locations and descriptions are presented in Chapter II. Nineteen sites were sampled on the LMR and its floodplain between river km 1031 and 998 representing various and varying degrees of connectivity with the main channel. These sites included the main channel, two side channels, a slack-water area along the main channel margin, seven relatively small floodplain lakes, and two large floodplain lakes sampled at multiple locations. Site connectivity was classified, from most to least connected to the main channel, as eupotamal, parapotamal, plesiopotamal, or paleopotamal in the same manner as Chapter II (classification from Ward and Stanford 1995). Briefly, eupotamal sites maintain a flow through connection year round, parapotamal maintain a downstream connection at average yearly low river stages, plesiopotamal only connect during average yearly high river stage but disconnect during lower stages, and paleopotamal only connect during flood events.

Samples were taken for metabarcoding twice in 2016, in May during high stage height/connectivity and October during low stage height/connectivity. Crustacean samples were collected by five vertical tows of 5 m length using a 153-µm mesh net to equal 283 L per sample. Rotifer samples were collected by passing 8 L of water, collected from 0.5 m below the surface, through a 32-µm mesh sieve. Each sample was preserved with 95% ethanol to a final concentration of ~70% and final volume of 50-mL. From each 50-mL concentrated sample, a 25-

mL subsample was used for DNA extraction. Here a subset of the original morphological data from Chapter II was used to match the two sampling dates of metabarcoding data.

3.2.3 DNA extraction and sequencing

Twenty-five mL of each zooplankton sample concentrate was centrifuged for 10 minutes at 9000 relative centrifugal force and the supernatant removed. Each sample was left to dry overnight to remove any additional ethanol. DNA was then extracted from dried tissue using DNeasy PowerSoil kits (Qiagen, Hilden, Germany). A 313 ± 10 bp region of the COI gene was amplified using forward (mlCOIintF, Leray et al. 2013) and reverse (jgHCO2198, Leray et al. 2013) primers in the two-round PCR process described by Clarke et al. (2017). However, for budgetary reasons only the 10 bp identifiers in the second round were used, forgoing the first round identifiers. For both rounds, DNA was initially denatured at 95°C for 10 min, then run through 35 cycles of denaturation at 95°C for 10 s, annealing at 46°C for 30 s, and elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. Three first round replicate PCRs were done on each DNA extraction. Each reaction mix contained 8 µL of AmpliTaq GoldTM 360 Master Mix, 0.5 µM each of forward and reverse primers, and 1 µL DNA extract for a final volume of 10 µL per reaction. First round PCR replicates were pooled then diluted 1:10 and Illumina sequencing adapters added in the second round of PCR using the same reaction quantities as the first round. The annealing temperature was increased to 55°C in the second round and cycles were reduced to 10 following Clarke et al. (2017). PCR products from each round were separated by gel electrophoresis and visualized on 1.5% agarose gels. Amplified DNA from the second round was normalized by sample using SequalPrep Normalization Plates (Life Technologies, Grand Island, New York), pooled, and paired-end

reads sequenced by the Molecular and Genomics Core Facility at the University of Mississippi Medical Center, Jackson MS, using an Illumina MiSeq.

3.2.4 Data analyses

Rotifers and crustaceans were analyzed separately. Demultiplexing of metabarcoding data was done by the Illumina MiSeq based on 10 bp identifiers. Primers were trimmed using Trimmomatic v0.36 (Bolger et al. 2014). Reads were merged with the -fastq_mergepairs command and low quality reads filtered with -fastq_filter (-fastq_maxee 1.0) in USEARCH v11.0.667 (Edgar 2010). Files were merged, into one file each for rotifers and crustaceans, with the make.group command and sequences screened using screen.seqs (start=2, end=303, maxambig=0, maxhomop=8) in mothur v1.41.1 (Schloss et al. 2009). Zero-radius operational taxonomic units (ZOTUs) were created and chimeras removed using the -fastx_uniques and -unoise3 commands and final OTU table created with the –otutab command in USEARCH v11.0.667 (Edgar 2010). ZOTUs are denoised (error-corrected) sequences that aim to represent true biological sequences (Edgar 2016). ZOTUs differ from 97% OTUs by not setting a hard similarity cutoff and attempt to distinguish all correct biological sequences that may be grouped together in traditional OTUs. Taxonomy was assigned to each ZOTU using MEGAN version 6.13.4 (Huson et al. 2016) using the top 20 BLASTN hits per ZOTU against the NCBI nt database (downloaded Dec. 12, 2018). Lowest common ancestor parameters were set to defaults, except Min support = 1, Min score = 300, Top percent = 10; these settings equate to a minimum of 80% similarity at full coverage. Both libraries contained a wide range of identified organisms (e.g. fish, insects, bacteria, mussels); however, only those ZOTUs assigned to Rotifera for the rotifer library and Crustacea for the crustacean library were retained for downstream analyses.

Species level taxonomy was retained for >95% identity at full coverage, even when species identification was recorded as a number after genus (e.g. *Anuraeopsis* sp. *WM-2017a*), otherwise they were reduced to family. Identifications by comparison with COI sequences in the NCBI database were checked against the BOLD database (Ratnasingham and Hebert 2007). However, many of the taxonomy listings that include numbers from NCBI have been incorporated into BOLD and I was unable to identify them with a specific epithet, especially within rotifers. Also, in some cases, BOLD was less likely to give a specific epithet because additional sequences in NCBI were not included in BOLD. BOLD assignments were used over NCBI assignments if they returned a "solid" match using the BOLD identification engine. However, very few rotifer assignments were changed in this manner, whereas crustacean assignments had several changes. Our most common crustacean ZOTU was assigned to the *Diaphanosoma* genus and not to a species. On inspection those ZOTUs were placed at genus because there were two entries in NCBI that refer to the same undescribed species (Dr. Manuel Elias-Gutierrez, personal communication) collected in two previous studies (Elias-Gutierrez et al. 2008; Garcia-Morales and Elias-Gutierrez 2013) that caused MEGAN to assign it only to genus level. ZOTUs assigned to the two entries were subsequently labeled *Diaphanosoma* sp. *1* in reference to the undescribed species from the two previously cited studies.

Statistical analyses were performed in R 3.5.1 (R Core Team 2018) using the packages vegan (Oksanen et al. 2018), ggplot2 (Wickham 2016), and Hmisc (Harrell and Dupont 2019). Random number generator seeds were set to "5" for all analyses. The main channel site was excluded from crustacean samples due to low sample size in morphological data. Beta diversity was quantified using the βsim presence/absence index (Lennon et al. 2002 based on Simpson

1943). βsim matrices were ordinated by nonmetric multidimensional scaling (NMDS) (Kruskal 1964).

3.3 Results

A total of 3,080,779 out of 3,631,447 reads were identified as Rotifera in the rotifer library and 2,167,052 out of 3,503,821 reads were identified as Crustacea in the crustacean library. There was not a statistically significant correlation between the number of reads compared to the number of ZOTUs in either rotifers ($r = 0.24$, $p = 0.14$) or crustaceans ($r = -0.15$, $p = 0.38$) (Figure 3.1). A total of 43 taxa, at all levels, were identified morphologically in the subset of samples from Chapter II and 82 taxa, at all levels, were identified with metabarcoding. Species level assignment differed greatly between the two methods with rotifers only sharing seven species and crustaceans only sharing three (Table 3.1). Of the 499 ZOTUs in the rotifer library, 198 or 40% were

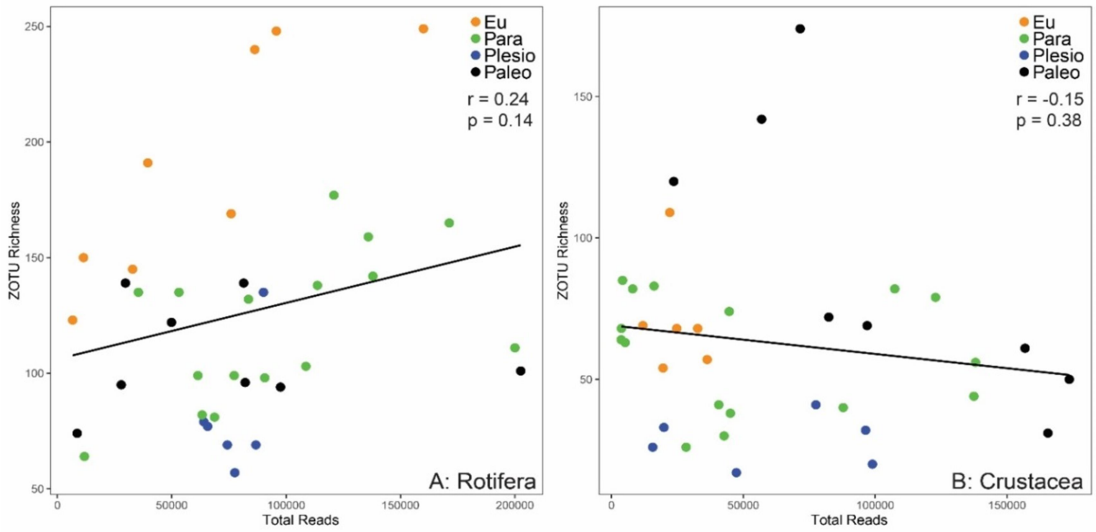

Figure 3.1. Correlations between total reads and ZOTU richness of zooplankton. Potamal state of sample sites is indicated by color.

Table 3.1. Richness of species level taxonomy assignments for morphology and metabarcoding datasets. Datasets include both spring and fall samples. Metabarcoding totals include species assignments that contain both entries labeled with numbers and entries labeled with specific epithet.

	Species Level Assignment		
	Morphology	Metabarcoding	Shared
Rotifera	25	23	7
Crustacea	6	16	3

assigned taxonomy at species level while 94 or 28% of 342 ZOTUs were assigned to species in the crustacean library. There was an average of 8.74 ZOTUs per species level taxonomy assignment in the rotifer library and an average of 5.87 ZOTUs per species level taxonomy assignment in the crustacean library. The top five most abundant rotifer taxa made up 79% of the total individuals per liter from the rotifer morphological dataset and the top five crustacean taxa made up 94% of total individuals per liter from the crustacean morphological dataset (Table 3.2). The top five most abundant rotifer ZOTUs made up 74% of the reads from the rotifer library and the top five crustacean ZOTUs made up 72% of the reads from the crustacean library (Table 3.3). Crustacean samples had a positive correlation between the number of morphological taxa and number of ZOTUs (Figure 3.2). However, rotifer samples were found to have a negative correlation between number of morphological taxa and number of ZOTUs.

Table 3.2. Taxon assignments and percentage of total individuals per liter for the top five taxa from Rotifera and Crustacea morphological datasets.

Dataset	Assigned Taxa	% of Total Ind./L
Rotifera	*Polyarthra remata*	24.4
	Anuraeopsis navicula	20.2
	Trichocerca pusilla	15.2
	Polyarthra vulgaris	10.8
	Brachionus caudatus	8.0
Crustacea	*Diaphanosoma* spp.	42.0
	Leptodiaptomus spp.	21.1
	Acanthocyclops spp.	15.4
	Daphnia parvula	8.2
	Bosmina longirostris	7.7

Table 3.3. Taxa assignments and percentage of total reads for the top five ZOTUs from Rotifera and Crustacea libraries.

Library	ZOTU #	Assigned Taxa	% of Total Reads
Rotifera	ZOTU 1	Polyarthra unclassified	54.9
	ZOTU 2	*Anuraeopsis sp. WM-2017a*	8.7
	ZOTU 3	*Anuraeopsis sp. WM-2017a*	3.5
	ZOTU 4	*Trichocerca sp. WM-2017e*	4.0
	ZOTU 5	Polyarthra unclassified	2.7
Crustacea	ZOTU 1	*Diaphanosoma sp. 1*	42.0
	ZOTU 2	Cyclopoida unclassified	21.1
	ZOTU 6	*Diaphanosoma sp. 1*	3.3
	ZOTU 7	Cyclopoida unclassified	2.8
	ZOTU 8	Cyclopoida unclassified	2.8

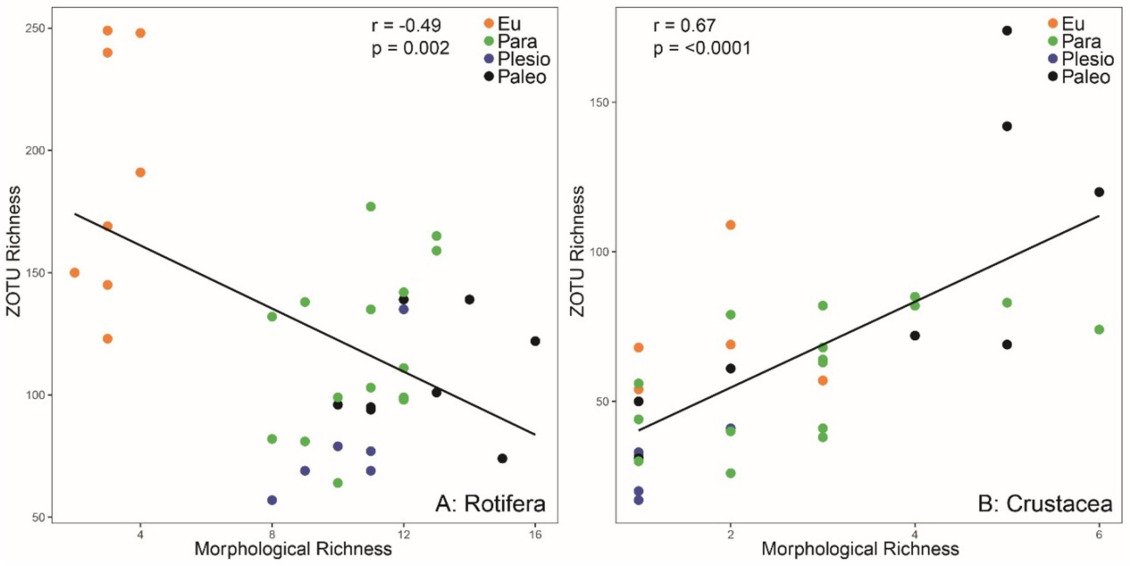

Figure 3.2. Correlations between morphological and ZOTU richness of zooplankton. Potamal state of sample sites is indicated by color.

In general, both total ZOTUs and total morphological taxa were lower in the fall compared to spring across all connectivity categories (Figure 3.3), with the exception of morphologically identified plesiopotamal rotifers. The highest richness was found in paleopotamal sites within the spring for three of the four datasets. Only rotifer metabarcoding had highest richness in eupotamal sites, although it was still in the spring sample. Rotifer morphological samples had the lowest richness in eupotamal sites followed by plesiopotamal and parapotamal with paleopotamal having the highest. Crustacean morphological samples had the lowest richness in plesiopotamal sites followed by eupotamal and parapotamal sites with paleopotamal again the highest. Both metabarcoding libraries found a decrease in ZOTU richness from eupotamal through plesiopotamal and an increase moving to paleopotamal regardless of sampling period. The largest discrepancy in patterns between morphological and metabarcoding richness datasets was comparing eupotamal sites. Morphologically identified rotifers had the lowest richness in eupotamal sites while these same sites had the highest ZOTU

richness. Crustacean eupotamal ZOTU richness was relatively even with parapotamal sites but lower compared to parapotamal in morphological richness.

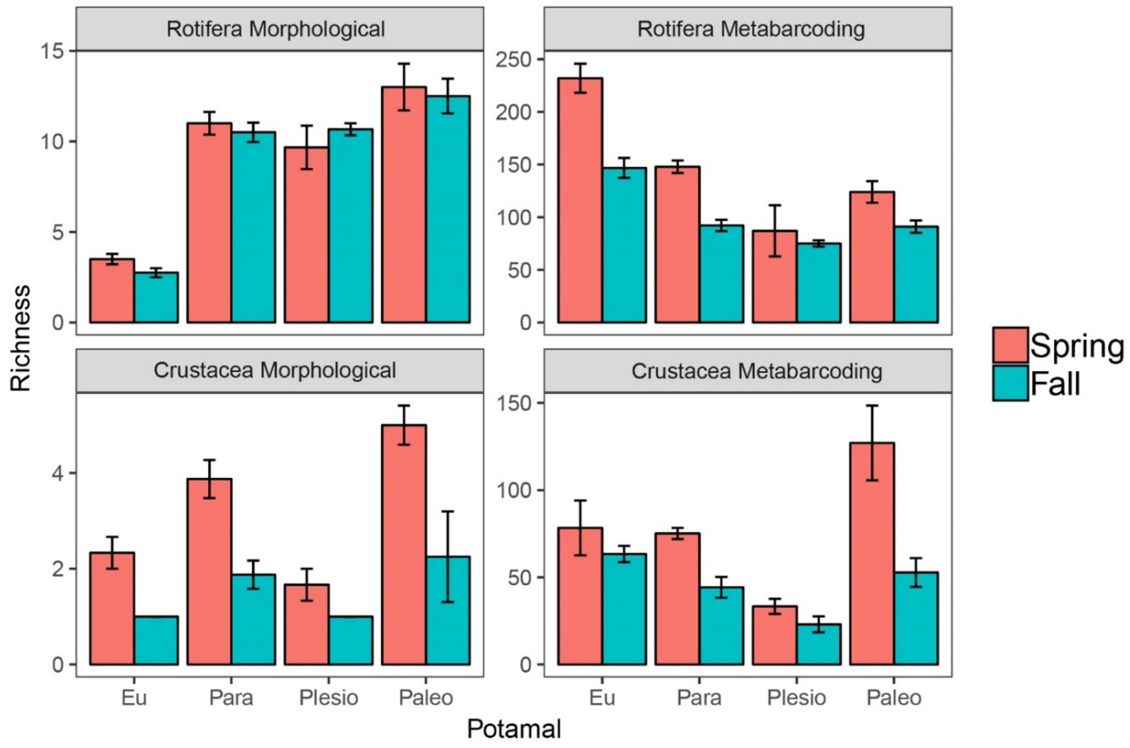

Figure 3.3. Comparison of richness of zooplankton between morphologically (taxa) and metabarcoding (ZOTU) identified samples in the Lower Mississippi River by potamal state. Bars represent mean ± standard error.

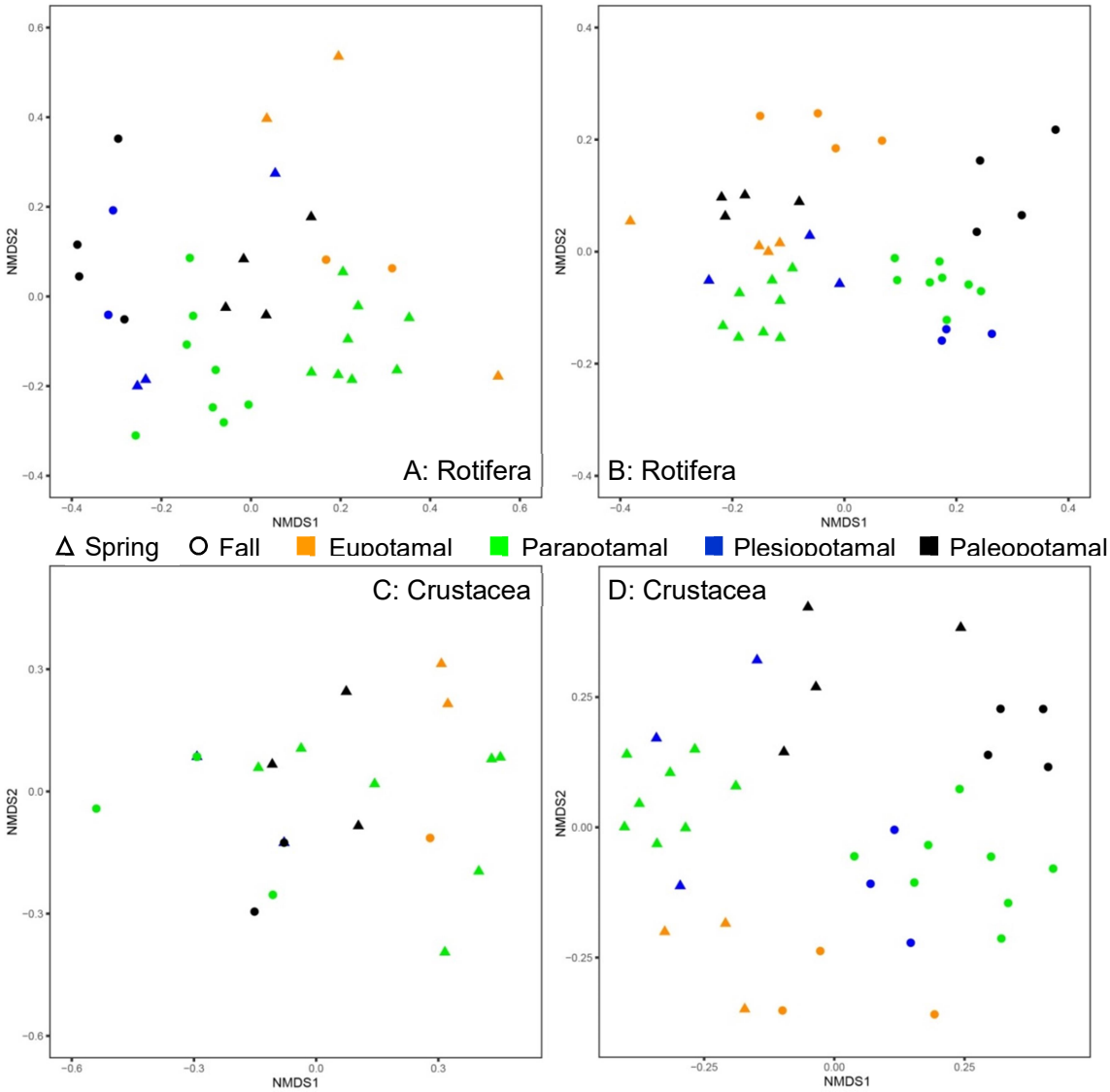

Figure 3.4. NMDS ordinations of rotifer and crustacean zooplankton across connectivity of the Lower Mississippi River. Ordinations represent beta diversity patterns based on βsim index for morphological (A and C) and metabarcoding (B and D). Shapes represent sampling period, colors represent potamal state.

Differences in beta diversity are represented in NMDS ordinations of the βsim index across ordination space (Figure 3.4). Both morphological ordinations (Figure 3.4A, C) had issues of overlapping samples (those that shared the same presence of taxa). However, neither metabarcoding ordination (Figure 3.4B, D) had overlapping samples. Rotifer morphological data (Figure 3.4A) show a separation between spring and fall sampling. However, eupotamal fall samples grouped with the majority of spring samples and two paleopotamal spring samples grouped with the majority of fall samples. Rotifer morphological data also had similar distances across connectivity as they did between sampling dates. Rotifer metabarcoding data (Figure 3.4B) show a clear separation of spring and fall samples. Fall samples had greater distances across connectivity than spring samples and there was a clearer grouping of connectivity categories between sampling dates in rotifer metabarcoding data compared to morphological data.

Crustacean morphological data (Figure 3.4C) suffered the most from overlapping samples with many samples indistinguishable from others. Very little patterning is evident within the ordination. Despite this issue, eupotamal sites grouped together as well as paleopotamal sites grouping together. No clear separation of sampling period is apparent from this ordination. Crustacean metabarcoding data (Figure 3.4D) display a visible separation in ordination space. Typically there was more distance across connectivity within a sampling period compared to within a connectivity category across sampling periods. Eupotamal sites had the least distance of all categories between sampling periods. As expected, two of the three plesiopotamal samples were in between parapotamal and paleopotamal within the spring sampling period. However, in the fall, plesiopotamal sites were closer to eupotamal compared to parapotamal sites. A single

spring paleopotamal sample grouped closer to the fall paleopotamal samples than the other spring samples.

3.4 Discussion

DNA metabarcoding of zooplankton assemblages has both positive and negative aspects. Metabarcoding found 39 more taxa than morphological identification, many at higher taxonomy than the species level. No correlation was found between morphologically-derived abundances and number of reads across samples, likely due to only counting adult crustaceans in morphological data and the additional biomass associated with egg production. Biomass increases have been shown to increase the number of reads produced from PCR (Krehenwinkel et al. 2017). Only slight overlap occurred at the species level between morphological and metabarcoding datasets (Table 3.1). The most likely reason for this discrepancy is the large amount of sequence entries without the inclusion of a specific epithet in the NCBI and BOLD databases. There were several ZOTUs in both metabarcoding datasets that were assigned to higher taxonomic levels because of high percent similarity between multiple reference sequences, some with specific epithets and some without. Also, many of the metabarcoding species assignments were to entries with numbers instead of specific epithets, especially in the rotifer dataset. Nevertheless, multiple additional species with specific epithet (six rotifers and nine crustaceans) were identified using metabarcoding compared to morphological identification. Some of these additions were likely not found morphologically because they occurred in low abundance, as, in fact, they were found and identified morphologically on sample dates not included in the metabarcoding analysis (*Brachionus quadridentatus*, *Daphnia ambigua*, and *Daphnia lumholtzi*). In the case of copepods, the four additional species level identifications are

due to morphological datasets being restricted to genus and metabarcoding's ability to identify larval individuals that are very difficult to identify morphologically.

In some instances ZOTUs can be assigned to the morphologically identified species. For example, ZOTUs assigned to *Anuraeopsis* sp. *WM-2017a* can realistically be considered to be *Anuraeopsis navicula* as this was the only *Anuraeopsis* assignment in morphological datasets. However, this limits comparisons as a positive identification for many ZOTUs is not possible. More species level assignments were found in rotifers morphologically than with metabarcoding, even when including numbered identifications. Again this is likely due to similar reference sequences being named differently. However, metabarcoding missed several taxa that were relatively highly abundant in particular samples, namely taxa within the Floscullariaceae order of rotifers. Floscullariaceae was largely absent from the NCBI database which precludes its discovery by metabarcoding analysis. Only a single, low-abundance, ZOTU was assigned to the Floscullariaceae order and it was restricted to the family Floscullariidae. Yang et al. (2017) found better agreement between morphological and metabarcoding identification; however, the authors supplemented the NCBI database with 70 morphologically identified taxa extracted individually and submitted to NCBI. These additional sequences are valuable for later studies using metabarcoding to identify zooplankton although Yang et al. (2017) were comparing metabarcoded zooplankton identified using their own morphological identifications to their morphological dataset. This process would be expected to show strong agreement between metabarcoding and morphological datasets.

On average, multiple ZOTUs were associated with a single species assignment; many of these ZOTUs were abundant in the libraries. Several known cryptic rotifer species had multiple

ZOTUs assigned to them, the most extreme examples of which were *Brachionus calyciflorus* with 14, *Polyarthra dolichoptera* with 10, and *Keratella cochlearis* with 101. Diversity within species and species complexes is indistinguishable within morphological datasets. Additionally, patterns in the most abundant morphological taxa were reflected in the most abundant ZOTUs (Table 3.2 and 3.3). Within rotifers, all of the most abundant taxa matched the most abundant ZOTUs except *Brachionus caudatus*, which was not included in the metabarcoding list. An unclassified Brachionidae ZOTU was relatively abundant but ranked eleventh in read abundance behind more ZOTUs identified as *Polyarthra* and *Trichocerca*. Within crustaceans, there is less agreement between the top morphological and metabarcoding taxa. However, the most abundant morphologically-derived taxon was also the most and third most abundant ZOTU assignments. The other three most abundant ZOTUs were all unclassified Cyclopoids which do not match morphological taxa, although the remaining morphological taxa line up with relatively abundant ZOTUs within the next ten ZOTUs.

Morphological and ZOTU richness of crustaceans were positively correlated (Figure 3.2B). However, there was an unexpected negative correlation between morphological and ZOTU richness in rotifers (Figure 3.2A). The negative correlation in rotifers was driven by low morphological richness compared to high ZOTU richness in eupotamal sites. Two potential explanations may clarify this discrepancy. First, rotifers are unable to resist lake outflows compared to large bodied crustaceans like *Daphnia* (Brook and Woodward 1956; Wicklum 1999) and are better able to survive turbid environments (Kirk 1991). Thus, rotifers that are morphologically identical but perhaps genetically differentiated are able to survive in the main channel, at potentially great distances from their origin population. Second, the high suspended

sediment of eupotamal environments makes morphological identification difficult, though not impossible. The difficulty in finding and identifying small rotifers within the main channel highlights a strong benefit to including metabarcoding in assemblage level research.

Beta diversity using metabarcoding libraries generally had greater separation of connectivity categories (Figure 3.4). Clear groupings can be seen in the metabarcoding ordinations compared to morphological ordinations. Both morphological datasets suffer from at least some overlap of samples due to the presence of the same species between samples. However, with additional resolution, metabarcoding ordinations are less confounded by overlapping samples. As expected, rotifer metabarcoding spring samples grouped together more than fall samples (Figure 3.4B). Connectivity increased during high river stage and homogenizes both environmental variables and zooplankton assemblages (Bozelli et al. 2015). Heterogeneity across the river system and between zooplankton assemblages increased as river stages decreased, which can clearly be seen in the rotifer metabarcoding ordination. The crustacean metabarcoding dataset does not display the same pattern (Figure 3.4D). This may be due to outflow avoidance by crustacean zooplankton, maintaining assemblage heterogeneity across connectivity at high river stage. Although homogenization of samples in spring was not as apparent as rotifers, differences in crustacean assemblages across connectivity by metabarcoding are more visible than in the morphological ordinations (Figure 3.4C). A single paleopotamal spring sample grouped closer to the fall paleopotamal samples than the other spring samples (Figure 3.4D). This sample was from the most disconnected of all sites making it the most stable environment within the sampling area. The stability of this site is possibly driving the

assemblage structure toward a later yearly succession sooner than the other paleopotamal samples.

3.5 Conclusion

The application of metabarcoding to analysis of river zooplankton assemblages found hidden diversity, additional rare species, and similar, although more defined, grouping and separation of groups from each other in beta diversity compared to discrimination by morphology. Metabarcoding revealed clear patterns in zooplankton assemblages across connectivity with only two samples per site, while morphological identification was less clear. Well-defined patterns in zooplankton assemblages across connectivity using metabarcoding are in part due to the speed and ease of analyzing metabarcoding samples compared to morphological samples. Morphological identification can take hours per sample leading to weeks if not months of processing whereas processing metabarcoding samples can be done in a fraction of the time. Eupotamal sites had a higher richness relative to less connected sites using ZOTUs compared to morphology and additional rare taxa were found across the river system using metabarcoding. Metabarcoding suffered the most from issues with database entries. In the case of rotifers, more species level identifications were found morphologically than with metabarcoding, likely driven by differently named reference sequences. A more curated database with the addition of missing taxa is necessary to use metabarcoding without using morphological identifications in tandem. Finally, until taxa-specific correcting factors are determined to adjust for primer and PCR bias, metabarcoding counts are unable to replace morphologically-based species abundances (Krehenwinkel et al. 2017). For these reasons, it is beneficial to continue to

use traditional morphology based identifications in conjunction with metabarcoding surveys until abundances can be determined using metabarcoding and issues with databases are resolved.

CHAPTER IV: SPATIAL DISTRIBUTION OF HAPLOTYPES AND CRYPTIC SPECIES DETECTION OF TARGET ZOOPLANKTON IN A LARGE RIVER SYSTEM

Abstract

Large rivers are temporally and spatially varied in connectivity between the main river channel and floodplain lakes creating distinct habitats. Zooplankton are unable to resist hydrologic flow and are thus mainly dispersed through above ground hydrologic connections between waterbodies. Many zooplankton species are cryptic species, which can coexist within the same discrete habitat. Given the high degree of connectivity between habitats of large river systems, I assessed the potential for the main river channel to act as a barrier to dispersal of zooplankton, and investigated the presence of cryptic zooplankton species within the Lower Mississippi River system. The COI gene was extracted and amplified from zooplankton species and haplotypes distinguished. Haplotype networks were constructed to examine intraspecific distribution. Three methods used (general mixed yule coalescent approach, Bayesian implementation of the Poisson tree process model, and automatic barcode gap discovery) to examine the presence of cryptic species Lower Mississippi River (LMR). Three species (*Anuraeopsis*

sp. *WM-2017a*, *Diaphanosoma* sp. *1*, *Leptodiaptomus siciloides*) were chosen to examine intraspecific dispersal patterns while two others (*Keratella cochlearis*, *Brachionus calyciflorus*) were chosen to examine the presence of cryptic species. *Anuraeopsis WM-2017a* was widespread and each haplotype was found in nearly all samples. *Diaphanosoma sp. 1* was more restricted than *Anuraeopsis WM-2017a*, although only a single haplotype was restricted to one side of the main river channel. *Leptodiaptomus siciloides* was the most geographically restricted species examined, found only in a select group of interconnected floodplain lakes. However, this species was restricted as a whole, with little spatial distribution of haplotypes. For cryptic species detection, *Keratella cochlearis* did not show agreement among the three methods used and thus presence of cryptic species cannot be determined with the data presented. Agreement was found among the three methods for *Brachionus calyciflorus* exhibited agreement, giving support for the presence of cryptic species. In light of the above I conclude that hydrologic connectivity does not necessarily equate to genetic connectivity, zooplankton are differentially affected by the river as a barrier to dispersal, and cryptic species of *Brachionus calyciflorus* are present in the Lower Mississippi River system.

4.1 Introduction

Zooplankton taxa were long thought to be cosmopolitan in their distribution, owing to small body size, large populations, and strong dispersal abilities. Zooplankton disperse passively through hydrologic above-ground connections, but are also known to disperse through animal, wind, and anthropogenic vectors (Havel and Shurin 2004). Although alternative methods for dispersal exist, Michels et al. (2001) found that genetic distances were better explained by hydrologic connections rather than Euclidean geographic distance between populations of

Daphnia ambigua. However, to our knowledge, no study to date has investigated the spatial distribution of genetic variants of zooplankton species across a large river floodplain.

The Lower Mississippi River varies temporally and spatially in discharge, depth, width, and habitat hydrologic connectivity of the main channel and floodplain lakes. Typically, all aquatic habitats within the levee system connect to the main channel during seasonal high water, although these connections do not necessarily occur every year and may be brief for the least connected aquatic sites. It is unknown whether this connectivity variation would produce genetic variation across the floodplain. However, it is likely that some intraspecific distribution restrictions would exist according to the monopolizing hypothesis of cyclically parthenogenetic zooplankton (De Meester et al. 2002; De Meester et al. 2007). The monopolizing hypothesis posits that locally adapted genotypes may negatively impact the establishment of immigrant genotypes through large egg banks, resulting in stark contrasts between dispersal ability and genetic variation even over short geographic distances.

Recent studies of both crustacean and rotifer taxa have also reported geographically structured cryptic taxa (Gómez et al. 2002; Belyaeva and Taylor 2009; García-Morales and Elías-Gutiérrez 2013; Bekker et al. 2016) and, in many cases, cryptic species have been shown to coexist simultaneously through divergence in responses to shifting physical environments and separation in diapause life-history traits (Montero-Pau et al. 2011; Gabaldón et al. 2017). Large rivers constitute a continuously shifting environment as the distinct habitats of the main channel and floodplain lakes connect and disconnect with fluctuating river stage. It is thus likely that cryptic zooplankton species are able to inhabit the landscape of large rivers, if not coexist within discrete habitats.

In light of the above observations I asked: 1) Are some zooplankton haplotypes restricted in their distribution in such a highly connected system? Although the main channel constitutes a hydrologic connection, the swift flow and large volume present a potentially impassible barrier to microscopic zooplankton within the relatively short sample section presented here. Thus, I hypothesized that the main river channel would be a barrier to zooplankton gene flow causing haplotypes to be restricted to one side of the river. 2) Do zooplankton species from three broad taxonomic groups (Rotifera, Cladocera, and Copepoda) show similar haplotype distribution patterns? Due to the high dispersal ability of the small bodied rotifers, I hypothesized that all haplotypes of sampled rotifer species would be broadly distributed, without any recognizable pattern, regardless of sampling period. I also hypothesized that haplotypes from cladoceran and copepod species would be more restricted than the rotifer, due to lake outflow avoidance by crustaceans (Wicklum 1999), with haplotypes occurring in fewer sampling sites especially during the low site to site hydrologic connectivity in the fall. 3) Finally I asked: Are cryptic species detectable within a morphological zooplankton species in the LMR? Given that some rotifer species are able to survive within the main channel of large rivers, I hypothesized that there would be evidence of cryptic species presence within a morphological species in the LMR. Rotifers able to survive the main channel conditions may be transported over long distances from their origin population, increasing the likelihood of cryptic species occurrence.

4.2 Methods

4.2.1 Zooplankton sampling

Nineteen sites were sampled in the LMR and its floodplain between river km 1031 and 998 (Figure 4.1) for rotifer species, while 18 sites were sampled for crustacean species. These

sites included the main channel (rotifers only), two side channels, a slack-water area along the main channel margin, seven relatively small floodplain lakes, and two large floodplain lakes sampled at multiple locations. Samples were taken in 2016, once in May during high river stage and once in October during low river stage. Rotifer and crustacean zooplankton were sampled separately. Rotifers were sampled by passing 8 L of 0.5 m depth subsurface water through a 32-µm mesh sieve. Crustaceans were sampled by five vertical tows of 5-m length using a 153-µm mesh net to equal 283 L per sample. Each sample was preserved with 95% ethanol to a final concentration of ~70% and final volume of 50-mL. From each 50-mL concentrated sample a 25-mL subsample was used in DNA extraction.

4.2.2 DNA extraction and sequencing

Twenty-five mL of each zooplankton sample concentrate was centrifuged for 10 minutes at 9000 RCF and the supernatant removed. Each sample was left to dry overnight to remove any additional ethanol. DNA was then extracted from dried tissue using DNeasy PowerSoil kits (Qiagen, Hilden, Germany). A 313 ± 10 bp region of the COI gene was amplified using forward (mlCOIintF, Leray et al. 2013) and reverse (jgHCO2198, Leray et al. 2013) primers in the two round PCR process described by Clarke et al. (2017). However, for budgetary reasons only the 10 bp identifiers in the second round were used, forgoing the first round identifiers. For both rounds, DNA was initially denatured at 95°C for 10 min, then run through 35 cycles of denaturation at 95°C for 10 s, annealing at 46°C for 30 s, and elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. Three first round replicate PCRs were done on each DNA extraction. Each reaction mix contained 8 µL of AmpliTaq GoldTM 360 Master

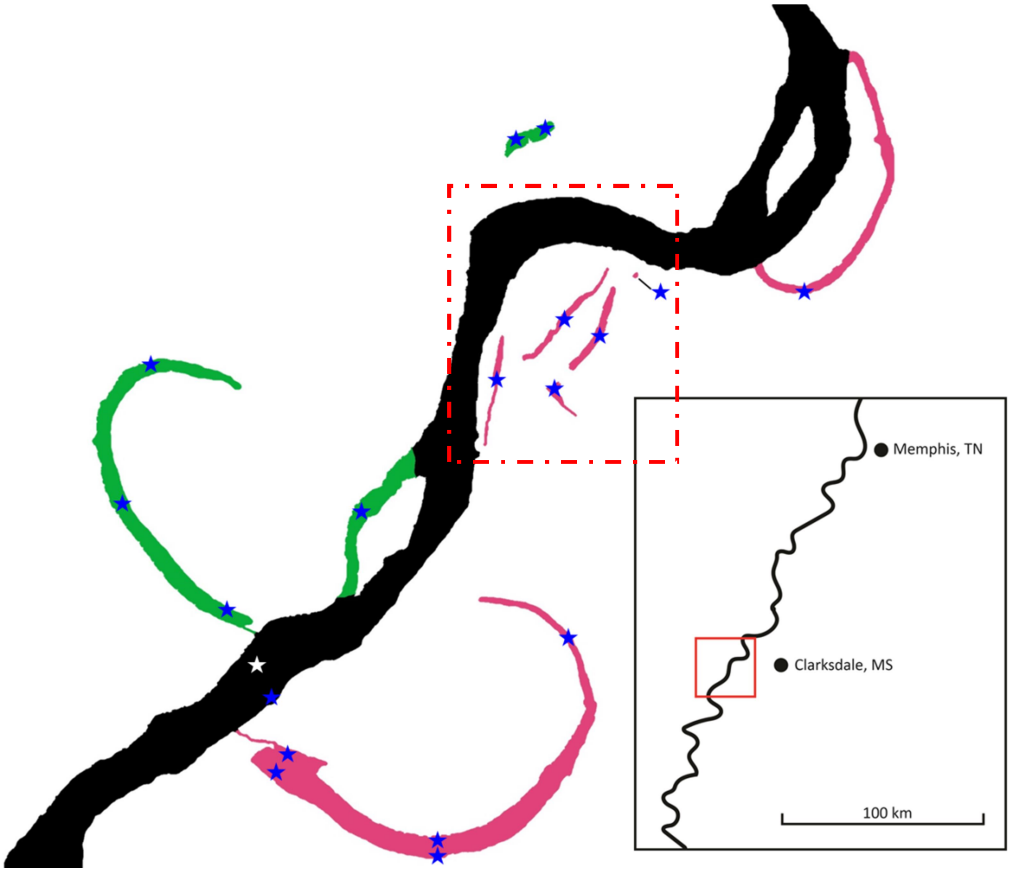

Figure 4.1. Map of LMR between river km 1031 and 998. Sample area in red box. Sampling sites as blue stars. White star represents the main channel which was excluded for crustacean samples. Green represents West river side sampling sites. Pink represents East river side sampling sites. Dashed square represents Burke's private hunting club.

Mix, 0.5 µM each of forward and reverse primers, and 1 µL DNA extract for a final volume of 10 µL per reaction. First round PCR replicates were pooled then diluted 1:10 and Illumina sequencing adapters added in the second round of PCR using the same reaction quantities as the first round. The annealing temperature was increased to 55°C in the second round and cycles were reduced to 10 following Clarke et al. (2017). PCR products from each round were separated by gel electrophoresis and visualized on 1.5% agarose gels. Amplified DNA from the second round was normalized by sample using SequalPrep Normalization Plates (Life Technologies,

Grand Island, New York), pooled, and sequenced by the Molecular and Genomics Core Facility at the University of Mississippi Medical Center, Jackson MS, using an Illumina MiSeq.

Demultiplexing was done by the Illumina MiSeq based on 10 bp identifiers. Primers were trimmed using Trimmomatic v0.36 (Bolger et al. 2014). Reads were merged with the -fastq_mergepairs command and low quality reads filtered with -fastq_filter (-fastq_maxee 1.0) in USEARCH v11.0.667 (Edgar 2010). Files were merged, into one file each for rotifers and crustaceans, with the make.group command and sequences screened using screen.seqs (start=2, end=303, maxambig=0, maxhomop=8) in mothur v1.41.1 (Schloss et al. 2009). Zero-radius operational taxonomic units (ZOTU) were created and chimeras removed using the -fastx_uniques and -unoise3 commands and final otu table created with the –otutab command in USEARCH v11.0.667 (Edgar 2010). ZOTUs are denoised (error-corrected) sequences that are aimed to represent true biological sequences (Edgar 2016). ZOTUs differ from 97% OTUs by not setting a hard similarity cutoff and attempt to distinguish all correct biological sequences that may be grouped together in traditional OTUs. ZOTUs were assigned taxonomy with MEGAN version 6.13.4 (Huson et al. 2016) using the top 20 BLASTN hits per ZOTU against the NCBI nt database (downloaded Dec. 12, 2018). Lowest common ancestor parameters were set to defaults, except Min support = 1, Min score = 300, Top percent = 10. Assigned taxonomy was checked against the BOLD database (Ratnasingham and Hebert 2007). Data analyzed here were also presented in Chapter III.

4.2.3 Spatial distribution of haplotypes

A representative species from each of the three major groups of zooplankton (Rotifera, Cladocera, and Copepoda) was chosen to test the hypothesis that haplotypes would be restricted

in their distribution. Criteria for representative species included: ZOTUs identified to species at minimum 95% similarity (checked against both NCBI and BOLD databases) and the most abundant species level assignment within each group from Chapter III. The three species chosen were *Anuraeopsis WM-2017a* (Rotifera, morphologically identified as *Anuraeopsis navicula* in Chapter III), *Diaphanosoma* sp. *1* (Cladocera), and *Leptodiaptomus siciloides* (Copepoda). These species are not meant to represent patterns in higher classifications as a whole and were partially chosen for convenience as the most abundant species assignments in their respective higher classification. ZOTUs were considered haplotypes in analyses. Haplotypes were retained if they made up >0.01% of total reads. To reduce potential cross-sample contamination during library preparation, haplotypes with fewer than five reads in a sample were reduced to 0 for that sample. The five read threshold was chosen after comparison of presence and absence of species between genetic data presented here and morphologically identified data presented in Chapter II. Retained haplotypes were then used to create statistical parsimony networks (Clement et al. 2000) using PopART v1.7 (Leigh and Bryant 2015). Sampling sites were color coded by whether they are on the East or West side of the river. The main channel was included as a third color in the rotifer network. When using metabarcoding data, potential primer bias across taxa is a concern, although using degenerate primers has been shown to reduce bias (Krehenwinkel et al. 2017). However, I have found no study examining within-species primer bias. Thus, for the sake of analysis, I make the assumption that haplotype read-abundance differences within a species are associated with biomass differences rather than primer bias. All other variables aside, it was expected that there would be an ~60:40 split between East to West in number of reads per haplotype given that sampling sites are split in the same manner.

4.2.4 Cryptic species detection

To test for the presence of cryptic species, I chose the known rotifer cryptic species complexes *Keratella cochlearis* (Derry et al. 2003) and *Brachionus calyciflorus* (Gilbert and Walsh 2005; Xiang et al. 2011). Both of these species are able to survive the harsh conditions of the main channel of the LMR and it follows that these species may travel long distances from their origin populations increasing the likelihood of cryptic species detection. Three techniques were implemented to detect cryptic species: generalized mixed Yule coalescent (GMYC) approach (Fujisawa and Barraclough 2013) using the splits package v1.0.19 (Ezard et al. 2017) in R v3.4.4 (R core team 2018), the Bayesian implementation of the Poisson tree process model (bPTP; Zhang et al. 2013) using the webserver at https://species.h-its.org/ptp/, and the automatic barcode gap discovery (ABGD; Puillandre et al. 2012) using the webserver at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html. These three techniques have been used previously for agreement cryptic species detection (Cieplinski et al. 2017). All rotifer ZOTU sequences produced after the taxonomy assignment step were used in analyses in order to overcome limitations due to low number of species, which can affect the ability of these techniques to accurately detect species. The phylogenetic tree used as input for both GMYC and bPTP was constructed using BEAST v2.5.2 (Bouckaert et al. 2019) using the following settings: log normal relaxed clock with a normalized molecular clock rate, substitution model was set using bModelTest (Bouckaert and Drummond 2017),the birth–death model, 10 million chain length with sampling every 1,000 trees. All sequences that were associated with class Bdelloidea were used as an outgroup as only this class and the class Monogononta were found. TreeAnnotator v2.5.2 was used to summarize trees and discard the first 2,000 as burn-in.

4.3 Results

4.3.1 Spatial distribution of haplotypes

A total of 442,249 reads were produced for *Anuraeopsis WM-2017a*, 1,033,779 reads for *Diaphanosoma sp. 1*, and 73,822 reads for *Leptodiaptomus siciloides*. After the strict final filtering, *Anuraeopsis WM-2017a* had 5 unique haplotypes with 8 segregating sites, *Diaphanosoma sp. 1* had 5 unique haplotypes with 6 segregating sites, and *Leptodiaptomus siciloides* had 8 unique haplotypes with 17 segregating sites. The East side of the river consistently had more reads of each haplotype.

All *Anuraeopsis WM-2017a* haplotypes were found across the landscape regardless of sampling period (Figure 4.2). Haplotypes increased in number of reads from spring to fall except for ZOTU 2. Fall samples were closer to the expected ratio than spring samples aside from ZOTU 2 which was near the expected ratio regardless of sampling period. The most abundant two haplotypes made up 87% of reads in spring and 83% of reads in the fall.

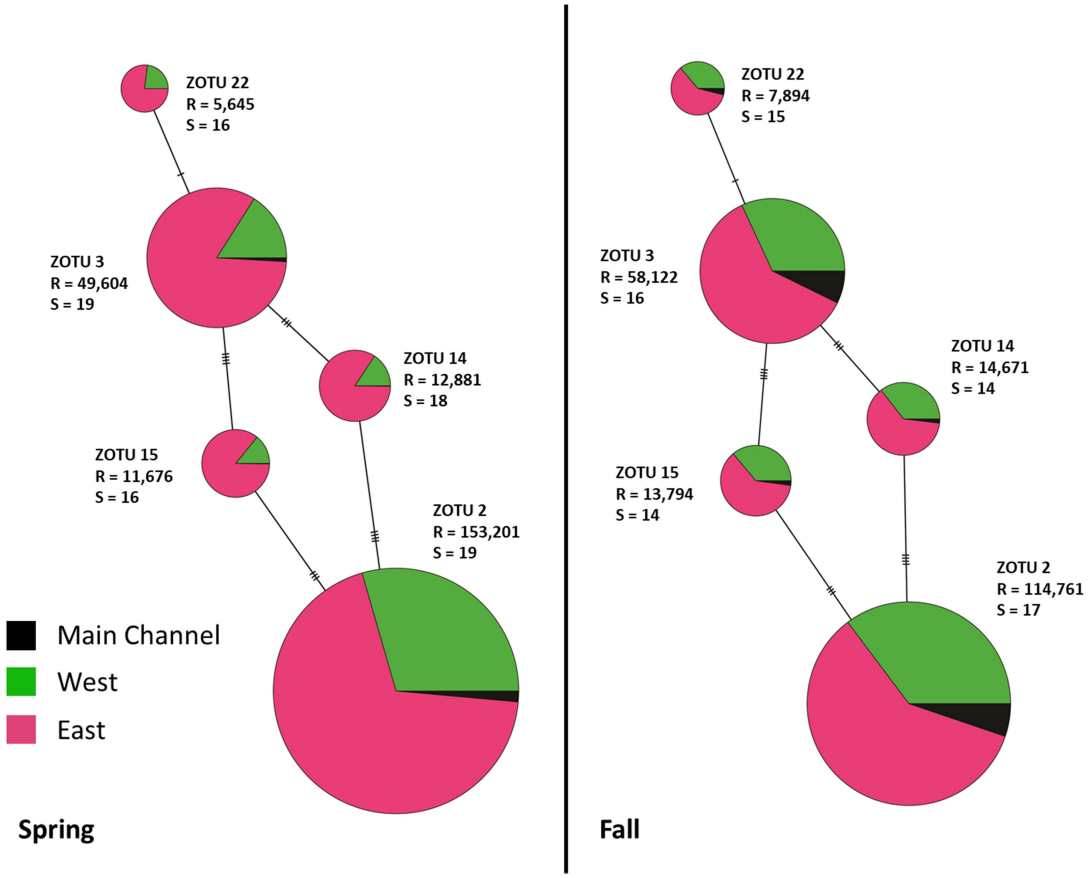

Figure 4.2. *Anuraeopsis WM-2017a* haplotype network based on 316-bp mitochondrial COI sequences for spring and fall sampling periods. Each circle represents a unique haplotype (ZOTU). R stands for number of reads for each haplotype and S stands for the number of sampling sites where each haplotype was found. Dashes along connecting lines indicate number of mutations, with each dash equating to one mutation. Size difference between circles represents relative number of reads. Sizes are relatable within sampling period only.

Diaphanosoma sp. 1 haplotypes varied in their distribution across the sampling region and across sampling periods (Figure 4.3). Between spring and fall, there was a large increase in the number of reads for each haplotype. During spring sampling, three haplotypes were restricted to the East side of the river, whereas in the fall only ZOTU 35 was restricted. The most common haplotype made up 88% of the reads in both spring and fall sampling periods.

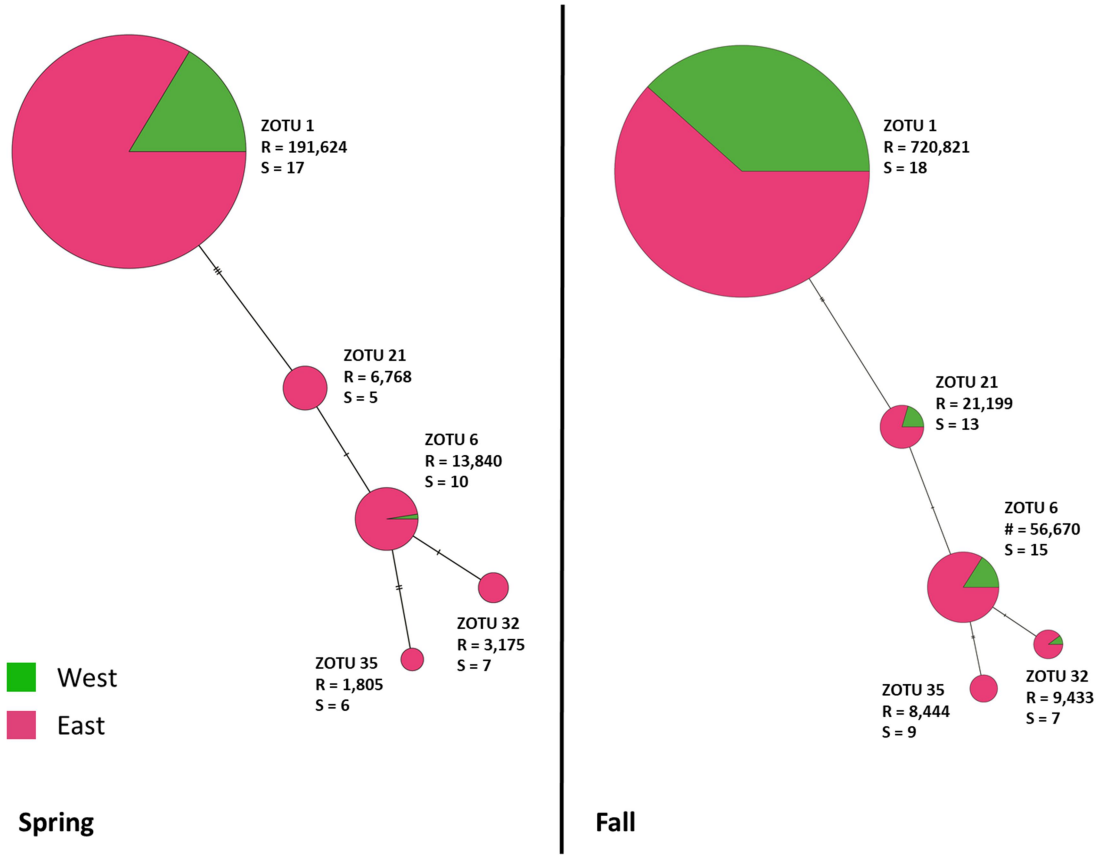

Figure 4.3. *Diaphanosoma sp. 1* haplotype network based on 313-bp mitochondrial COI sequences for spring and fall sampling periods. Each circle represents a unique haplotype (ZOTU). R stands for number of reads for each haplotype and S stands for the number of sampling sites where each haplotype was found. Dashes along connecting lines indicate number of mutations, with each dash equating to one mutation. Size difference between circles represents relative number of reads. Sizes are relatable within sampling period only.

Leptodiaptomus siciloides was the most restricted species examined. Most of the haplotypes were restricted to the East side of the river in the spring (Figure 4.4). Two haplotypes had a few reads on the West side of the river but were only found in one sampling site each. By fall, only three haplotypes remained and they were restricted to a single site on the East side of

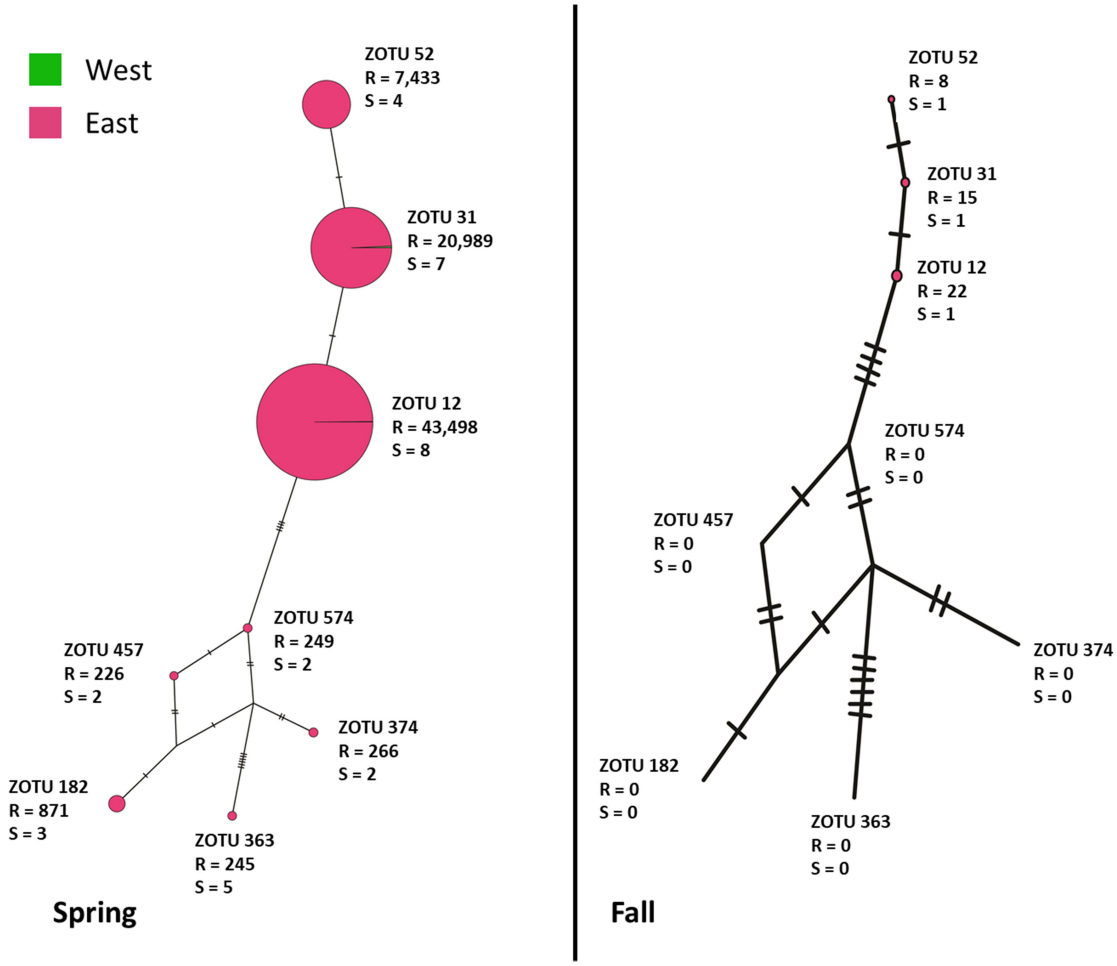

Figure 4.4. *Leptodiaptomus siciloides* haplotype network based on 313-bp mitochondrial COI sequences for spring and fall sampling periods. Each circle represents a unique haplotype (ZOTU). R stands for number of reads for each haplotype and S stands for the number of sampling sites where each haplotype was found. Dashes along connecting lines indicate number of mutations, with each dash equating to one mutation. Size difference between circles represents relative number of reads. Sizes are relatable within sampling period only.

the river and had only 45 total reads. The top two haplotypes made up 87% of the reads in the spring.

4.3.2 Cryptic species detection

For *Keratella cochlearis*, GMYC indicated three separate groups of representative sequences (Figure 4.5). The first consisted of 96,347 reads from 59 ZOTUs, the second of 89,390 reads from 38 ZOTUs, and the third of 516 reads from 3 ZOTUs. The results of

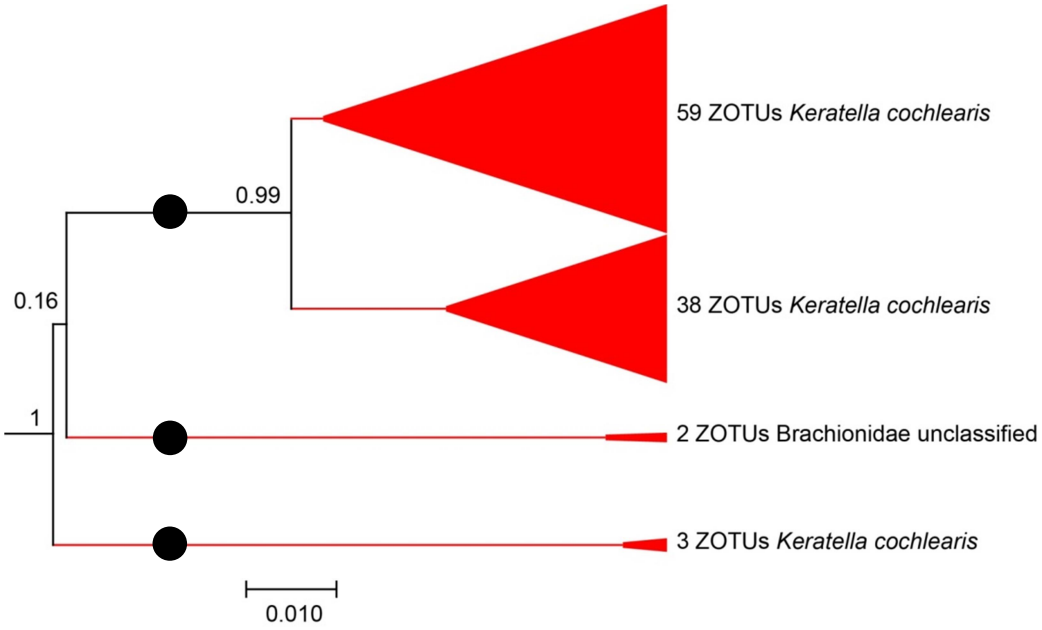

Figure 4.5. Results of *Keratella cochlearis* species detection by GMYC and bPTP. Red cones signify groups of ZOTUs determined by GMYC. Black dots signify groups determined by bPTP. ABGD not included as all sequences that were taxonomically assigned to *Keratella cochlearis* were grouped as a single species. Bayesian posterior probabilities are included above nodes. Branch length proportional to the number of substitutions per site.

bPTP showed two groups instead of three, combining the first and second groups from the GMYC approach (Figure 4.5 black dots). Finally, the ABGD approach combined all ZOTUs

assigned to *Keratella cochlearis* into a single group. Brachionidae unclassified included as part of phylogenetic tree.

For *Brachionus calyciflorus*, GMYC indicated six separate groups of representative sequences (Figure 4.6). The first consisted of 1,338 reads from 5 ZOTUs, the second of 59 reads from 1 ZOTU, the third of 2,286 reads from 4 ZOTUs, the fourth

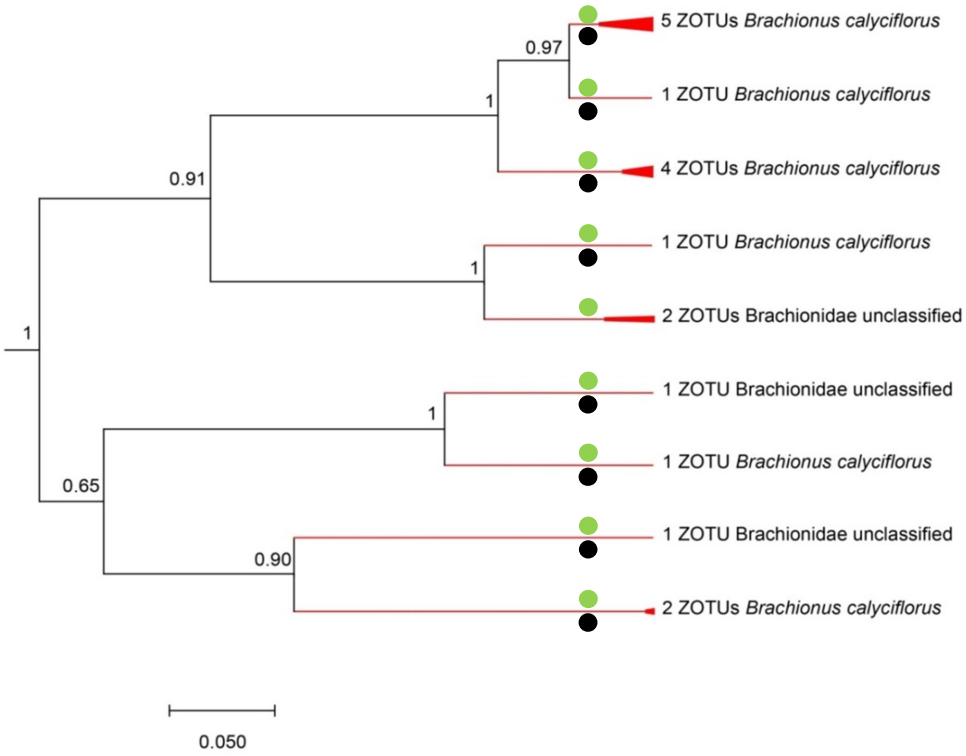

Figure 4.6. Results of *Brachionus calyciflorus* species detection by GMYC, bPTP, and ABGD. Red branches/cones signify groups of ZOTUs determined by GMYC. Black dots signify groups determined by bPTP. Green dots signify groups determined by ABGD. Bayesian posterior probabilities are included above nodes. Branch length proportional to the number of substitutions per site.

of 21 reads from 1 ZOTU, the fifth of 2,003 reads from 1 ZOTU, and the six of 254 from 2 ZOTUs. The results of both bPTP and ABGD agreed with those of GMYC (Figure 4.5 black and

green dots). The only difference in results was that the bPTP method separated two ZOTUs assigned to Brachionidae unclassified that were grouped in the other two methods.

4.4 Discussion

4.4.1 Spatial distribution of haplotypes

For all three zooplankton examined for haplotype distribution, I found multiple haplotypes across the study area. As expected, none of the five haplotypes of *Anuraeopsis WM-2107a* were restricted in their distribution regardless of sampling period (Figure 4.2). *Anuraeopsis* species are known to be abundant in tropical and sub-tropical areas along with temperate regions when conditions are favorable (Wallace et al. 2006). High abundances combined with small size and inability to resist hydrologic flow likely give *Anuraeopsis WM-2107a* its high dispersal ability. Finding all haplotypes across the sampling area suggests that this species is not restricted by the main river channel. However, there was a reduction in the number of sampling sites in which haplotypes were present in the fall compared to spring sampling. Haplotypes were similarly excluded, or drastically reduced in number of reads, from four sites between spring and fall. From the data I collected, there is no discernable explanation as to why those sites lost *Anuraeopsis WM-2017a*.

The cladoceran species, *Diaphanosoma* sp. *1*, was more restricted in its haplotype distribution compared to *Anuraeopsis WM-2017a* (Figure 4.3). This species was more restricted to one side of the river in spring sampling compared to fall. *Diaphanosoma* sp. *1* haplotypes also occurred in more sampling sites in the fall compared to spring sampling, opposite our hypothesis. Zooplankton time their emergence from resting stages for optimal conditions (Wallace and Snell 2010). Our results suggest that less dominant haplotypes within a species, those that may be less

adapted to local conditions in accordance with the monopolizing hypothesis, are also more restricted in emergence timing than dominant haplotypes which were found across the landscape regardless of sampling period. Thus, although *Diaphanosoma* sp. *1* appears spatially restricted in the spring sampling, they are actually temporally restricted for all but one haplotype. The single haplotype (ZOTU 35) was restricted to the east side of the river regardless of sampling date. This haplotype was mainly found within the Burke's hunting club (Figure 4.1 red dashed square). The lakes found within the hunting club are the oldest floodplain sites sampled. If this haplotype is a mutation, it follows that the most likely area for it to occur is in the oldest sampling sites as mutations are infrequent and more time equates to more mutations. Likewise, if the haplotype is an immigrant, it follows that the haplotype would be detectable in the oldest sites where enough time has passed to escape the persistent founder effects of the dominant haplotype (De Meester et al. 2007). Either of these explanations is plausible and would clarify why this *Diaphanosoma* sp. *1* haplotype is not found on the west side of the river, giving support to the hypothesis that the main channel is a barrier to genetic distribution within the levee system.

The final species examined for spatial distribution of haplotypes, *Leptodiaptomus siciloides*, was the most restricted (Figure 4.4). However, the restriction seen in *L. siciloides* was for the species as a whole and not between haplotypes. This species was almost restricted to the east side of the river with only 2/8 haplotypes occurring in one site each on the west side of the river and only represented by a small number of reads. Like ZOTU 35 from *Diaphanosoma* sp. *1*, *L. siciloides* was mainly restricted to the older lakes of the Burke's hunting club. However, this is likely due to strict environmental conditions necessary for *Leptodiaptomus siciloides* to survive as a species instead of mutations or immigration since no haplotype is more restricted

than the others. Further evidence of environmental constraints is represented by the near loss of the species during the fall sampling period. Only three haplotypes remain in the fall and they were all found in a single site.

4.4.2 Cryptic species detection

There were mixed results for cryptic species detection of two known cryptic species of rotifers. For *Keratella cochlearis*, there was not agreement across the three methods (Figure 4.5). Both GMYC and bPTP showed multiple sequence groupings, but ABGD grouped all *Keratella cochlearis* sequences together. Without agreement between the three methods, it is impossible in this study to say there is evidence for cryptic species within *Keratella cochlearis*. However, this species had the most ZOTUs associated with it than any other species level assignment by far (Chapter III). With such a large number of sequence variants within the species, limitations may be attributed to the relatively short reads used in the analysis. The possibility of errors produced during PCR or sequencing cannot be discounted, although the next most ZOTUs associated with a single species was 18, meaning that errors were species specific.

For *Brachionus calyciflorus*, all three methods indicated the same groups of sequences, providing support for the presence of cryptic species. There were no discernable patterns in habitat differentiation between groups of *Brachionus calyciflorus*. However, multiple means of cryptic species coexistence have been observed for rotifers including resource use and vulnerability to predation (Gabaldón et al. 2017), response to fluctuating environments (Montero-Pau et al. 2011) and density dependent investment in sex and diapause (Montero-Pau and Serra 2011). All are potential explanations for coexistence in the present study area. *Brachionus calyciflorus* was mainly found in the two large lakes sampled (Figure 4.1); these

lakes maintain a downstream connection to the main channel of the river and near continuous fluctuating environments. The other modes for coexistence are also plausible; however, I did not examine their potential in the current study. A still unanswered question is whether the indicated *Brachionus calyciflorus* cryptic species are predominantly native to the sampling area or are immigrants being transported by the main channel.

4.5 Conclusion

The LMR is a highly connected system on a year to year basis and little spatial distribution of haplotypes of zooplankton would be expected, given their high dispersal potential. However, taking all results of spatial distribution of haplotypes into account, the main channel is likely a barrier to zooplankton dispersal; however, this depends on the species being examined. The small rotifer species with high dispersal potential compared to larger species was found nearly everywhere regardless of sampling period, whereas the two crustacean species were more restricted. Only one species examined (*Diaphanosoma* sp. *1*) displayed haplotype distribution restriction with a single haplotype being found on one side of the main river channel. Cryptic species were also found within this study within the morphological species *Brachionus calyciflorus*. However, our expectation was to also find cryptic species within *Keratella cochlearis,* a species able to survive and dominate the main channel. In fact, the evidence of presence of cryptic species occurred in a species mainly restricted to two large floodplain lakes. These cryptic species are likely coexisting; however, additional research is needed to determine the means of their coexistence. This study supports the hypotheses that zooplankton are differentially affected by habitat connectivity, that hydrologic connectivity does not necessarily

confer genetic connectivity, and that the cryptic species *Brachionus calyciflorus* has coexisting variants within the LMR.

CHAPTER V: CONCLUSION

The hydrologies of the world's largest rivers have been extensively researched in order to perform large scale engineering for human use. Relatively little research has focused on the biological and ecological aspects of these large rivers. The influence of hydrologic connectivity on organism assemblage structure in river systems has recently become a focus of river scientists (Hein et al. 2001; Pringle 2003; Harrison et al. 2017). Zooplankton, which are unable to resist flow, are strongly affected by hydrologic connectivity with the main channel and assemblage structure is governed by the frequency of connection to floodplain habitats (Baranyi et al. 2002; Goździejewska et al. 2016, Balkić et al. 2018). However, these recent studies were conducted on much smaller and less hydrologically dynamic river systems compared to the LMR and few studies have examined zooplankton in the LMR in general.

Our first objective was to establish which environmental variables differed between four potamal connectivity categories and, because the LMR is such a large system, if large floodplain lakes exhibited differences in environmental variables. I found that there were five environmental variables (TDN, Turb, Chla, Depth, and DO) that were important in distinguishing spatial connectivity and one (Temp) that was highly correlated with season. Of these variables, TDN, Turb, and Chla varied across large

oxbow lakes. Further, upstream regions of large lakes were less affected by high river stages than plesiopotamal lakes indicating the large lakes may contain a gradient in connectivity, one that remains connected to the main channel but confers that connection over a large volume and great length.

After establishment of differing environmental characteristics, I was interested in how zooplankton assemblages respond to spatially and temporally varying main channel connectivity and the environmental correlates of zooplankton assemblages. Rotifer abundances, as expected, were reduced during and shortly after connection events which is in line with previous studies (Baranyi et al. 2002). In contrast to previous studies, crustaceans in the two most connected habitats increased in abundance during high connectivity periods. The major terrestrial habitat of the LMR is forested (Baker et al. 1991) and, when flooded, is an ideal place for crustacean zooplankton to inhabit and reproduce (Flinn et al. 2005; Górski et al. 2013). It follows that crustaceans would then be forced out of these prime habitats and into the main channel and more connected floodplain habitats by hydrologic transport during high river stage. Most zooplankton occurred across the landscape, but individual taxa took advantage of one connectivity category over others where they increased in their share of total abundance. One of the most correlated environmental variables to zooplankton assemblages was turbidity. Our results matched those of the Missouri River (Thorp and Mantovani 2005) more closely than those of the Upper Mississippi and Ohio rivers (Thorp and Mantovani 2005; Burdis and Hoxmeier 2011), all of which are tributaries to the LMR. These results indicate that zooplankton assemblages are strongly affected by suspended sediments in large river systems. These results together stress the importance of variably-connected habitats for zooplankton diversity and maintenance of

zooplankton assemblages. Historically, the natural meandering of the LMR created new habitat and altered the connectivity of existing habitat, but this process has been greatly reduced over recent history. Sediments continue to fill floodplain habitats (Hudson et al. 2008), and it is likely that engineering will be necessary to preserve or restore aquatic habitat heterogeneity in the future.

A difficult aspect of zooplankton research is the identification process, typically done using morphological characteristics. Metabarcoding has shown promise as an alternative method of identification in marine and freshwater systems (Clarke et al. 2017; Yang et al. 2017). However, before the current study, metabarcoding had not been used to analyze zooplankton across a gradient in hydrologic connectivity and compare results to morphology based identification. Previous studies also sequenced individual zooplankton identified morphologically and used them in the reference database for metabarcoding identification (Yang et al. 2017). Mixed results were found for metabarcoding identification. Metabarcoding found additional taxa not found morphologically, several of which were likely in low abundance causing them to be missed in the smaller morphological sample. Metabarcoding also found hidden diversity within species represented by multiple ZOTUs identified to the same species. Finally, patterns across connectivity were better defined using metabarcoding in the limited sampling done in this part of the study. However, without proper taxa specific correcting factors, true individual abundances are not possible with metabarcoding (Krehenwinkel et al.2017). Considering the above results, both morphology and metabarcoding are best used in tandem until issues with metabarcoding are resolved.

Bearing in mind that there are assemblage differences throughout the LMR, it follows that there may also be spatial distribution of haplotypes within the study area. Zooplankton disperse mainly passively through above-ground hydrologic connections (Michels et al. 2001; Havel and Shurin 2004) and the LMR typically connects to all extant floodplain habitats yearly during high river stages. This study is the first to examine spatial distribution of haplotypes of zooplankton across a large river system. The three species examined exhibited differing distributional patterns. The rotifer species, *Anuraeopsis WM-2107a*, was widespread and showed little haplotype distributional restriction. The large number of individuals (discussed in Chapter II) and abundant reads (Chapter III), along with small size and inability to resist slight hydrologic flow, suggest that *Anuraeopsis WM-2107a* have high dispersal ability across the scale of this study. Few *Anuraeopsis* were found within the main channel potentially limiting their dispersal ability (Chapter II). However, comparable read abundance between the main channel and floodplain habitats (Chapter III) appear contradictory to morphologically identified samples. One potential explanation would be the presence of resting eggs being transported without adults, increasing distribution potential. The cladoceran species, *Diaphanosoma sp. 1*, had a single haplotype restricted to one side of the main river channel and largely within a set of lakes that are the oldest floodplain formations. If this haplotype is either a mutation or an immigrant, it is probable that the variant would be found within the oldest lakes as enough time would pass for mutations to occur or for an immigrant to overcome the monopolizing hypothesis of De Meester et al. (2007). The third species, *Leptodiaptomus siciloides*, was the most restricted as a whole species with no haplotype distributional patterns. This species may have the most habitat restrictions, limiting it to the oldest/least connected floodplain lakes. The above results indicate

that spatial distribution of haplotypes patterns are present in the highly connected LMR system and that the main river channel is a restriction to intraspecies dispersal. However, this conclusion is highly dependent on the species being examined.

Finally, I investigated the presence of cryptic species of two rotifer species. Rotifers have been shown to cryptic species separated geographically (Gómez et al. 2002; Bekker et al. 2016) and ecologically by habitat preference and separation in life-history traits (Montero-Pau et al. 2011; Gabaldón et al. 2017). The LMR system is continuously shifting with river stage and, as shown in Chapter II, has differing habitat structure related to spatial variation in hydrologic connectivity. It follows that cryptic rotifer species would be found across the LMR if not coexist within individual floodplain lakes. No agreement was found between cryptic species detection method for *Keratella cochlearis*, suggesting either a single species occurs in the LMR or the COI region used is not variable enough to detect cryptic speciation. For a second species, *Brachionus calyciflorus*, all three methods agreed on six potential cryptic species. Habitat differentiation was not detected between these six groups; however, as stated above, coexistence between cryptic rotifer species is common.

Zooplankton are an important piece of overall ecological function as grazers, predators, prey, and nutrient cyclers (Vanni 2002). My research contributes to the growing body of knowledge revealing the assemblage structure and dynamics of this ecologically significant group of organisms across riverine systems. Continued research is necessary to determine to fully understand assemblage and population dynamics of zooplankton. For instance, the relative contributions to assemblage structure of individuals hatched from egg banks and those emigrating from other areas are unknown. Also, little is known of the species level contributions

to ecological function, as zooplankton are typically studied as a group. Although continued work is necessary, it is clear that zooplankton assemblages of large river systems are controlled by variables coincident with the pattern and degree of hydrologic connectivity.

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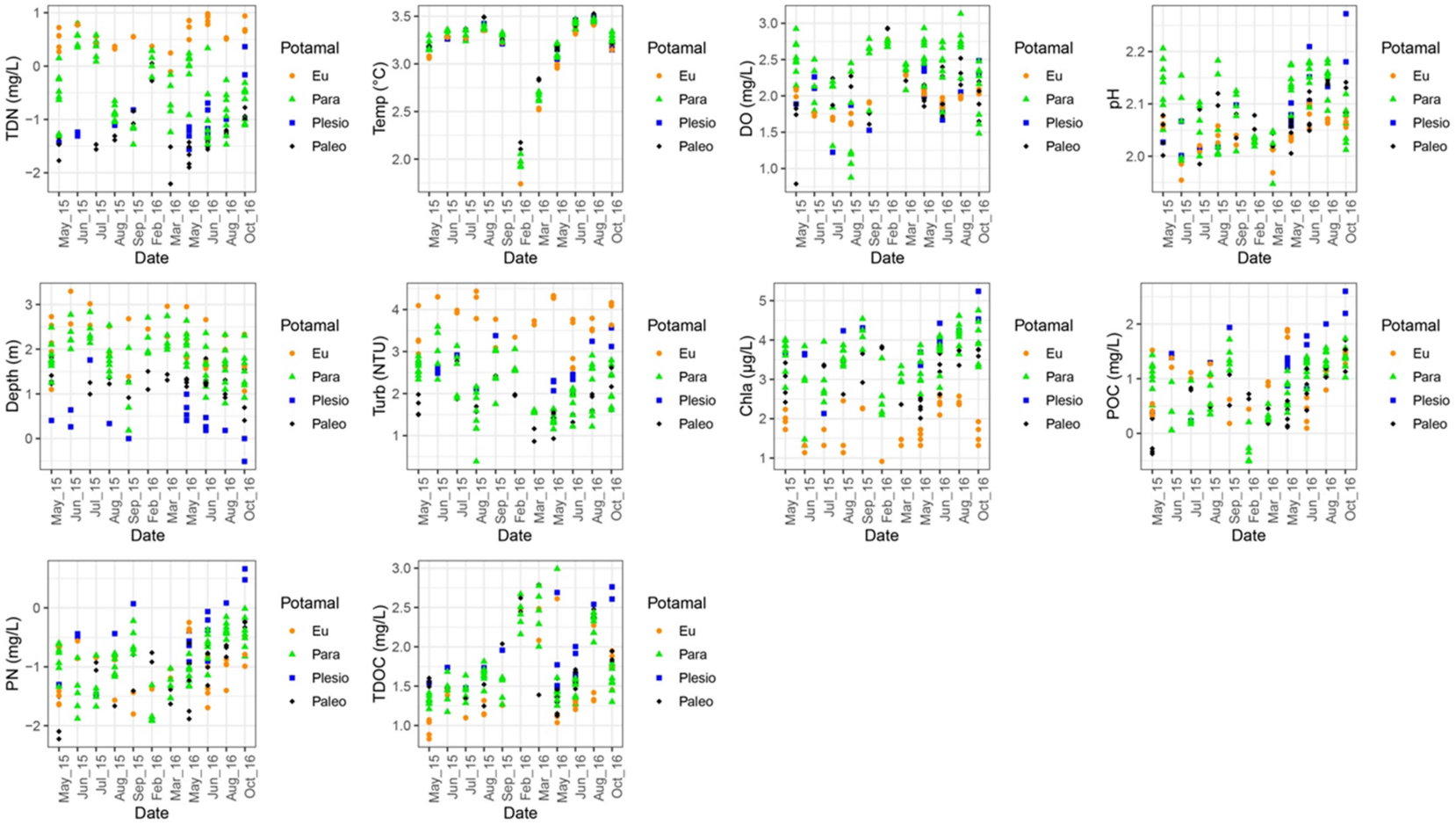
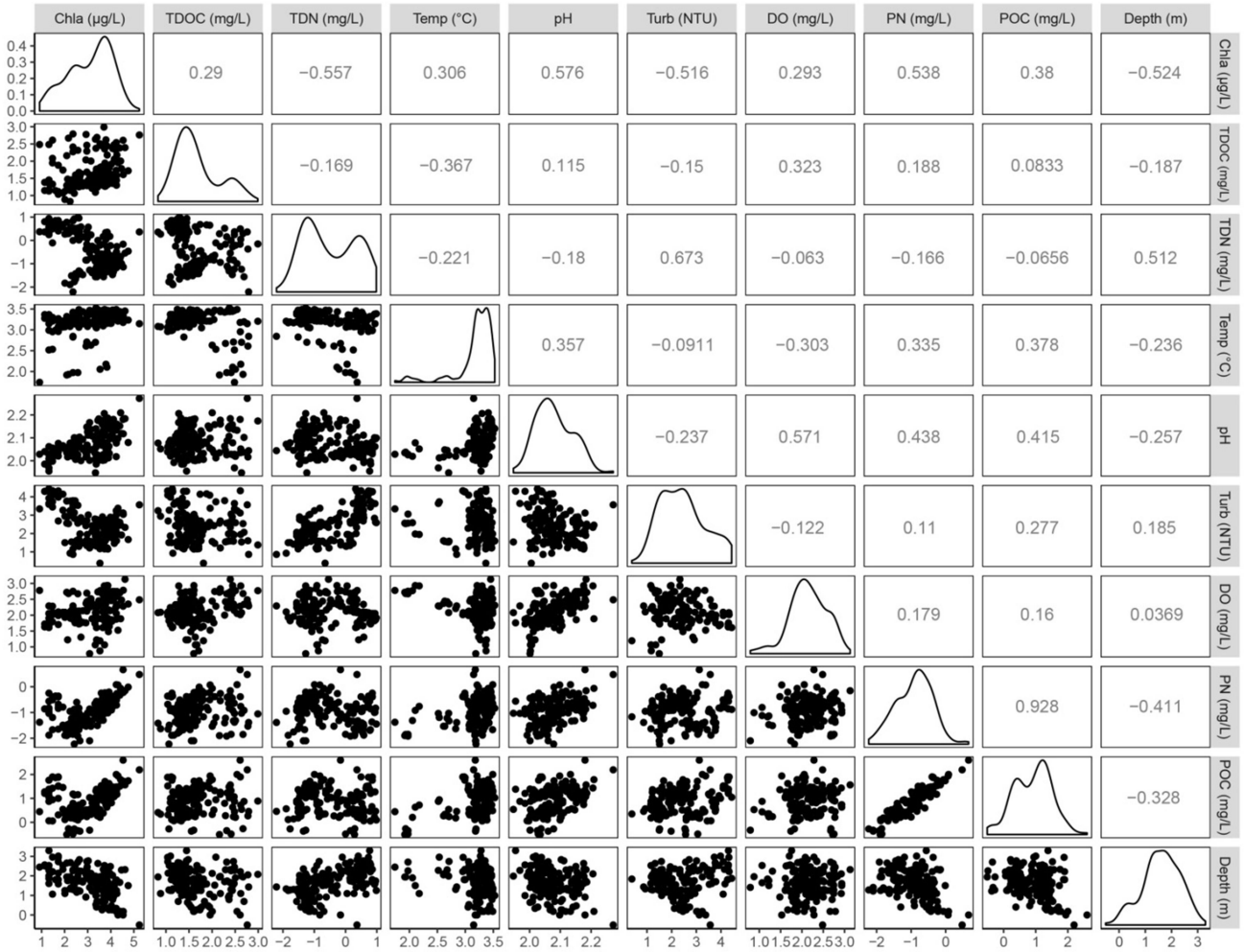
LIST OF APPENDICES

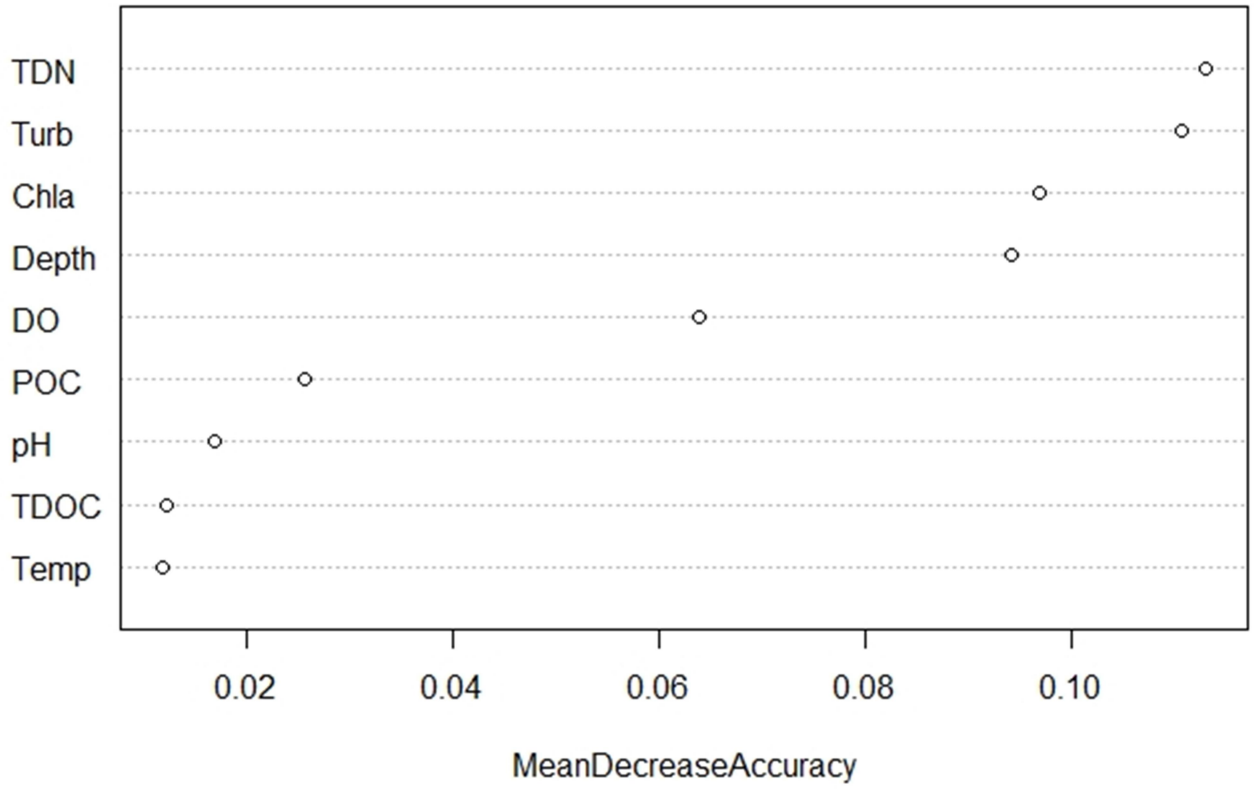
Appendix A. Environmental variables over sampling period for all sites. All values are log transformed. Color coded for connectivity category.



Appendix B. Pairwise correlation of environmental variables. All variables are log transformed. Pearson correlation coeficient in upper right. Data from all samples from 2015-2016. Units on x-axis match top variables, units on y-axis match right variables.



Appendix C. Results from randomForest, mean decrease in accuracy with permutation of each variable across a decision tree.



VITA

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Education

Winona State University, Winona
Bachelor of Science in Biology – Environmental Science 2007 - 2012

Presentations

2018 Sackreiter, J., C.A. Ochs, and K.J. Killgore. August, 2018. Spatial and temporal variation in main channel connectivity drive zooplankton assemblage dynamics in the Lower Mississippi River floodplain. Ecological Society of America Annual Meeting, New Orleans, LA. (oral)

2017 Sackreiter, J., C.A. Ochs, and K.J. Killgore. November 2017. The Influence of Site Connectivity on Zooplankton Assemblage Dynamics within the Lower Mississippi River Floodplain. International Society for River Science meeting, Hamilton, New Zealand. (oral)

2015 Sackreiter, J., Ochs, C.A. Hydrologic connectivity as a driver of zooplankton community structure across a large river floodplain. International Society for River Science, La Crosse, Wisconsin – USA (oral)

2014 Sackreiter, J., Ochs, C.A. Zooplankton assemblages of the Lower Mississippi River in relation to connectivity. Joint Aquatic Sciences Meeting, Portland, Oregon – USA (poster)

2012 Sackreiter, J., Delong, M.D., & Richardson, W.B. Zooplankton community dynamics across a large river mosaic. Mississippi River Research Consortium, La Crosse, Wisconsin – USA (poster)

Publications in preparation

Sackreiter, J., C.A. Ochs, C. Murphy, and K.J. Killgore. (submitted to Freshwater Biology) Hydrologic connectivity as a driver of temporal and spatial variation in zooplankton assemblage structure across a large river floodplain

Sackreiter, J., C.A. Ochs, C. Murphy, and K.J. Killgore. Trophic position of zooplankton assemblages in relation to connectivity using stable isotopes

Sackreiter, J., C.A. Ochs, C. Murphy, and K.J. Killgore. Comparison of DNA metabarcoding and morphological identification of zooplankton assemblages across a large river floodplain

Sackreiter, J., C.A. Ochs, C. Murphy, and K.J. Killgore. Spatial distribution of haplotypes patterns and cryptic species detection of target zooplankton in a large river system

Silwal, S., D. Padmanava, J. Sackreiter, C.A. Ochs, J.L. Pinckney, and R. J. Moorhead. Determination of phytoplankton community structure in multiple water bodies and comparison of the potential of several in situ and laboratory techniques.

Awards

University of Mississippi Summer Fellowship. 2018 & 2019

Research assistant/research support on external grant - Evaluation of Biogeochemical Processes in the Lower Mississippi River System. US Army Corps ERDC Coop Agreement W912HZ-15-2-0012 (awarded to C. Ochs). 2015 – 2017

Research assistant - MRI: Acquisition of an Imaging Flow Cytometer for multidisciplinary organic and inorganic particle research and education. PIs: C.A. Ochs, R. Buchholz, T. Goulet, J. Hoeksema. Granting agency: NSF grant DBI-1126379. 2015

Research assistant - Nitrogen removal in Mississippi Delta agricultural streams related to flow rate, productivity and metabolic mode: Experiments using stream mesocosms. USDA-ARS NSL WQE specific cooperative agreement with the UMFS/CWWR. (Awarded to C.A. Ochs). 2013

Teaching Experience

University of Mississippi, Oxford
Teaching Assistant 2012 – Present
Introductory Biology I & II (8 semesters)
Introduction to Aquatic Biology (1 semester)
General Ecology (1 semester)

Related Experience

Field Work

- Sampling of aquatic plants and animals by boat

- Communication with private land owners
- Knowledge of farming practices (from family farm)
- Electro shock sampling (both back-pack and boat)
- Collection of aquatic environmental variables

Laboratory work
- Laboratory analysis of chemical constituents
- Particle analysis using, and maintenance of, Fluid Imaging Technologies FlowCam
- Stable Isotope sample preparation
- Organic carbon and nitrogen analysis using, and maintenance of, Shimadzu Total Organic Carbon Autoanalyzer
- DNA extraction and PCR of individuals and assemblages of aquatic invertebrates
- Compound microscopy including the use of differential interference contrast
- Identification of organisms using dichotomous keys

Computer skills
- R, PRIMER, JMP, GIS, MOTHUR, USEARCH, MEGAN, MEGA
- Familiar with a wide variety of univariate and multivariate analysis techniques (e.g. ANOVA, PERMANOVA, machine learning, linear mixed effects models, genetic analysis techniques)

Outreach
- Demonstrated aquatic sampling and organisms to middle school students as part of the Lower Mississippi River Foundation
- Demonstrated aquatic sampling and organisms during a field trip for high school students from Helena, AR